HAEMOLYSIS BY NEWCASTLE DISEASE VIRUS

II. GENERAL CHARACTER OF HAEMOLYTIC ACTION

by F. M. BURNET AND P. E. LIND[1]

(From the Walter and Eliza Hall Institute of Medical Research, Melbourne).

(Accepted for publication 10th November, 1949.)

Although haemolysis has been observed in this laboratory with mumps and Newcastle disease viruses for some years, its occurrence was irregular and its degree always very incomplete. The finding recorded in a footnote by Morgan, Enders and Wagley (1949) that cells, rendered insusceptible to mumps virus agglutination by the action of influenza viruses, resisted haemolysis, stimulated a systematic study of NDV in the hope that this would throw light on the general nature of virus action on the cell surface.

The outstanding impression that has emerged from the work has been the variability and lability of the haemolytic activity of the virus. The activity of different batches of allantoic fluid has varied greatly and independently of their haemagglutinin titres. With some preparations the haemolytic activity was much more strongly inhibited by the addition of Ca ions than with others. When virus was precipitated by methanol in the cold some extremely active preparations were obtained but even with meticulous care completely reproducible results could not be guaranteed. Some showed very rapid haemolysis, others an initial lag period of variable length. Untreated virus regularly was more active on fowl than on human erythrocytes, methanol-precipitated virus in almost every instance was more active against human cells.

Nevertheless, despite this variability, there is a general uniformity of behaviour which marks off virus haemolysis (mumps and NDV) from any other type of haemolysis. The important features of NDV action are: Haemolytic activity is destroyed by heating to 54° C., or higher, haemolysis is insignificant below a temperature in the range of 22°–28° C., the reaction ceases at a certain level of haemolysis depending essentially on the virus/cell ratio, no haemolysis results with cells which have been treated so that they cannot adsorb NDV particles and finally haemolysis is inhibited by agents which can block the haemagglutinating action of NDV (immune serum and periodate-treated ovomucin).

[1] This work was aided by a grant from the National Health and Medical Research Council, Canberra.

In this paper these more general characters of the reaction will be described. A more detailed study of methanol-precipitated virus will be presented in a subsequent paper of the series.

MATERIALS AND METHODS.

The virus strains used and the general methods for production of viruses, haemagglutination tests, etc. have been described in the preceding paper. We are indebted to Mr. G. L. Ada for the following description of the method based on that of Cox *et al.* (1947) by which the methanol-precipitated virus used in many of the experiments was prepared. A full account of the influence of various factors on the yield of virus and its haemolytic activity will be given in a subsequent paper.

Preparation of Methanol-Precipitated Virus.

Methanol, cooled to —50° C. with dry ice, is cautiously added to final concentration of 30 p.c. to NDV allantoic fluid, previously cooled to 0° C. During the addition, the temperature of the solution is allowed to fall to —5° C., and is kept at this temperature for 3 hours after addition is completed. The solution is then centrifuged at 3,500 r.p.m. for 15 minutes at —5° C., the resulting clear supernatant discarded, and the sediment taken up in 10 p.c. of the original volume of physiological saline, and dialysed against saline for 24 hours at 0° C. Any undissolved material is then removed by centrifugation.

Estimation of Haemolytic Activity.

The most commonly used procedure was to follow the course of haemolysis by taking samples at intervals from mixtures held at 37° C. Final volumes of 5 ml. were generally used, the virus dilutions being brought to 37° C. before the final addition of 1/10 the volume of a 10 p.c. suspension of red cells. At the required intervals one ml. samples were chilled in ice water and centrifuged as rapidly as possible. The supernatant fluid was removed at once and when the experiment had been completed the haemoglobin content of the fluids was estimated in the photoelectric colorimeter described by Holden (1946). In many sighting experiments and in a few of those reported here, standard tubes corresponding to 50 p.c. and 25 p.c. haemolysis of the red cell suspension being used were prepared and the degree of haemolysis in the experimental mixtures compared with these. In preliminary titrations of the haemolytic activity of MPV preparations it was convenient to use as endpoint the final dilution of virus in phosphate saline which gave 25 p.c. haemolysis of human cells after 1 hour at 37° C.

Determination of Receptor Status of Treated Cells.

In some experiments cells treated with the receptor destroying enzyme (RDE) of *V. cholerae* were used. Depending on circumstances either the crude extract prepared as described by Burnet and Stone (1947) or purified material was used. The method of purification involved adsorption to and elution from red cells and ammonium sulphate precipitation (Ada, French, Curtain and Laver). Cells treated either with RDE or viruses were examined for their receptor status with the same series of viruses as was used by Burnet, McCrea and Stone (1946) and Stone (1947) in defining the "receptor gradient." The following viruses were included: Mumps (Enders strain), NDV, MEL, WSE, LEE.B, BEL.A, SW and MIL.B. all of which have been described in the papers referred to. The cells were appropriately treated to remove as far as possible the virus or enzyme with which they had been initially treated and a 1 p.c. suspension in saline tested with 5 agglutinating doses (for normal cells of the same species) of each virus. A control tube with normal saline to detect any spontaneous agglutinability of the cells was always included.

EXPERIMENTAL.

1. *General Character of the Haemolysis by NDV.*

Haemolysis by allantoic fluid virus is slow and incomplete, its extent varies considerably from one sample to another and is not regularly proportional to the haemagglutinin content of the fluid. Haemolysis is more marked against fowl than against human cells. Eluates prepared by adsorption on red cells in the cold with elution into a smaller volume of saline at 18–20° C. (not at 37° C) behave similarly. Amniotic fluids from embryos inoculated by this route may show very high haemagglutinin titres and proportionately more active haemolysis.

By far the most actively haemolytic preparations are obtained by precipitation of the virus from allantoic fluid by methanol in the cold followed by resuspension and dialysis. The preparation of methanol - precipitated virus (MPV) has been the work of Mr. G. L. Ada and a full description of the method and the implications of the phenomenon will be published in collaboration with him. In this paper the results obtained with typical MPV preparations will be used to illustrate the general character of the phenomena.

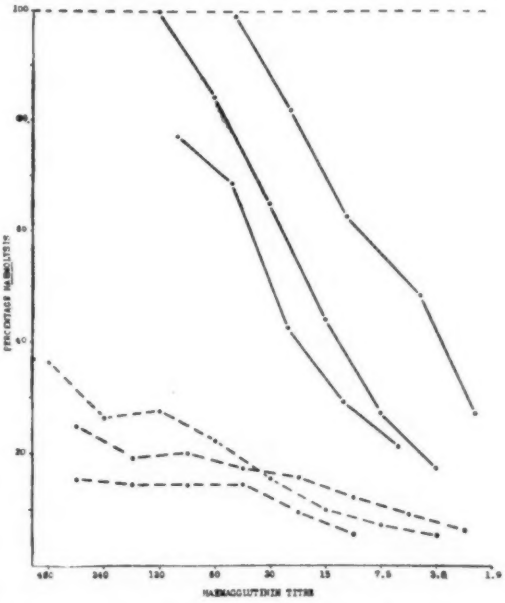

Fig. 1. Haemolytic titration curves for 3 allantoic fluids (3 hours at 37° C.) (broken lines) and 3 methanol-precipitated virus preparations (1 hour at 37° C.) (solid lines).

Ordinates: Percentage haemolysis of 1 p.c. suspension of human cells.

Abscissae: Concentration of virus in terms of haemagglutinating titre, logarithmic scale.

Two general types of experiment have been used to characterize the haemolytic activity of different preparations. (1) Dilutions of the fluid are made in 1 ml. volumes of normal saline buffered with phosphate at pH 7·35. These are warmed to 37° C. and then 0·25 ml. of 5 p.c. red cells (human or fowl as required) added. After 1 hour with MPV preparations or 3 hours with unprecipitated material the tubes are centrifuged and the haemoglobin in the supernatant estimated either in the photoelectric colorimeter or more usually against standard tubes showing 50 and 25 p.c. haemolysis. When a single figure is desired to express activity the final virus dilution giving 25 p.c. haemolysis is taken as the haemolytic titre.

(2) Suitable dilutions of virus in phosphate saline are warmed to 37° C., human or fowl cells added to give a final 1 p.c. suspension and samples taken at appropriate intervals. These are chilled, spun rapidly and the content of haemoglobin estimated in the supernatant fluid by photoelectric colorimeter.

In Fig. 1 are shown haemolytic titration curves for three allantoic fluid viruses and three MPV preparations the degree of haemolysis being plotted against the concentration in terms of haemagglutinating doses. The haemolysis by the allantoic fluids would be almost negligible at 1 hour so that a 3-hour period is used for these.

The results with MPV at 3 hours would have shown some increase in the degree of haemolysis by the higher dilutions but would not have greatly changed the form of the curves. It will be noticed that irrespective of the activity of the more concentrated preparations the curves all tend to reach zero haemolysis at about the same point near 1 haemagglutinating unit.

Several examples of curves showing the time course of haemolysis are used to illustrate experiments described later (Figs. 2, 4 and 5). The most important points of difference to be noted are the varying character of the initial stages, the speed at which the more or less constant final level is reached and the absolute height of that level.

2. *The Species Range of Cells Haemolysed.*

All detailed work has been confined to human and fowl cells. The work was initiated with fowl cells since these are agglutinated by NDV to much higher titres than human cells and they are more susceptible to haemolysis by untreated virus. The stromata (plus nuclei) resulting from lysis are much more readily handled than human cell stromata and for work concerned with stromata they have been used exclusively. All haemagglutinin titres mentioned have been estimated with fowl cells used at a final dilution of 0·5 p.c. and allowed to settle at room temperature.

Human cells are more readily and rapidly lysed by MPV and have been used for most of the work on the kinetics of haemolysis. They are also more convenient for estimations of changes in electrophoretic mobility.

TABLE 1.

Comparative activity of MPV 16A on different species of red cell.

Species	Haemagglutinin titre	Haemolytic titre
Human	280	140
Guinea pig	400	100
Rabbit	<10	<10
Sheep	200	180
Horse	100	10
Ox	400	160
Mouse	100	160
Fowl	800	40
Pigeon	520	140

Haemolytic titre = dilution producing 25 p.c. haemolysis of 1 p.c. cells in 1 hour at 37° C.

Most of the other readily available types of cell have been tested against typical MPV preparations. Table 1 gives the haemagglutinin and haemolytic titres (25 p.c. haemolysis of 1 p.c. cells) obtained against MPV 16A with cells from representative species. It will be seen that most are susceptible to haemolysis, the two exceptions being for rabbit and horse cells. Rabbit cells are neither agglutinated nor haemolysed, horse cells are poorly agglutinated and show only weak haemolysis by the virus.

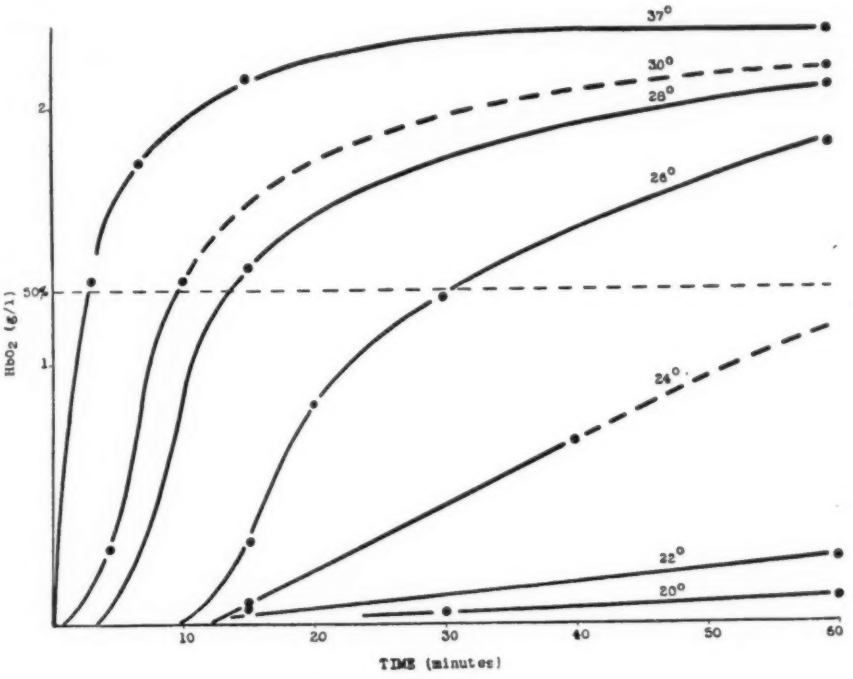

Fig. 2. The course of haemolysis of human cells by MPV at temperatures from 20° C.–37° C.

Ordinates: Haemoglobin gm./l. in supernatant fluids.

MPV Batch 2 used at a final dilution of 1:20 equivalent to 60 haemagglutinating doses, 1 p.c. human cells in phosphate saline.

3. *Inactivation by Heat.*

In the first paper of this series (Burnet, 1950) it has been shown that when allantoic fluid virus is heated to 54° C. or higher for 30 minutes haemolytic activity disappears in parallel with the ability of the virus to make firm union to red cells. This holds also for the much more active methanol-precipitated virus. When this is diluted in phosphate saline all activity is lost at 54° C., with undiluted material two samples showed some residual haemolytic activity after heating at 54° C., none after 56° C. Haemagglutinating activity and enzymic action against ovomucin are not significantly reduced at either temperature.

4. *Temperature in Relation to Haemolysis.*

At temperatures below 30° C. the rate of haemolysis by MPV rapidly diminishes and at room temperature 17–18° C. there is only a trace of haemolysis after 20 hours. Haemolysis curves obtained at different temperatures with one batch of MPV and human cells are shown in Fig. 2. From these curves it is possible to obtain the velocity of haemolysis during the initial period when the rate of liberation of haemoglobin is approximately constant. The velocity for each temperature expressed at gm./l. HbO_2 liberated per hour is shown in Table 2.

In the course of these experiments the unhaemolysed cells were washed with saline and tested for stability in normal saline and for the effect on their stability of dilute immune fowl serum. The results have already been given in Paper I of this series but are included again for convenience of reference. There is a sharp appearance of the irreversible type 2 reaction at the temperature at which haemolysis becomes significant.

With fowl cells the rate falls more rapidly below 28° C. than with human cells.

TABLE 2.

Action of MPV on human cells at different temperatures.

Temperature	Velocity of haemolysis	Agglutination with	
		N.S.	I.F.S.
17–18° C.	0·009	+	—
20° C.	0·11	+	—
22° C.	0·24	+	—
24 ° C.	1·5	—	+
26° C.	3·7	—	+
30° C.	13·4	—	+
37° C.	20·0	—	+

5. *Time and Concentration Relationships of Haemolysis.*

The most interesting feature of the haemolytic curves is the appearance of a flat terminal portion indicating that haemolysis virtually ceases at a certain level. By suitable tests of supernatant fluid and cells it is easy to show that this cessation of haemolysis is due not to exhaustion of haemolytic activity but to the development of resistance to haemolysis by the cells. If attention is for the time being confined to the final level of haemolysis the conditions determining the level reached may be stated as follows:

1. The level depends on the type of virus preparation used being much higher for MPV than for untreated allantoic fluid virus of equivalent haemagglutinating titre.

2. To a fair approximation over a moderately wide range of concentrations the final level is proportional to log concentration of virus.

Both these points can be exemplified in Fig. 1 in which the concentration of virus (expressed in haemagglutinating units) is shown logarithmically.

3. The relationship between extent of haemolysis and cell concentration with constant amounts of virus, varies according to the reagents used. With allantoic fluid virus and fowl cells the degree of haemolysis is closely proportional to the concentration of cells, i.e. there is an approximately uniform percentage haemolysis. With the less sensitive human cells the percentage haemolysis is highest with the highest virus/cell ratio. Results of a typical experi-

ment are shown in Table 2. With MPV there is again no simple relationship between cell concentration and percentage haemolysis. Figs. 3 and 4 represent results from a single experiment in which MPV Batch 2 of haemagglutinin titre 800 was tested at final dilutions of 1:10, 20, 40, 80 and 160 against 1 p.c. fowl cells and at 1:20 dilution against 0·5, 1·0, 2·0 and 5·0 p.c. cells.

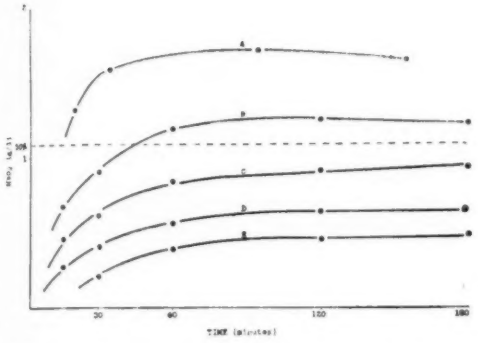

Fig. 3. Course of haemolysis of 1 p.c. fowl cells with varying amounts of MPV. Final dilutions of virus MPV Batch 2. A 1:10, B 1:20, C 1:40, D 1:80, E 1:160.

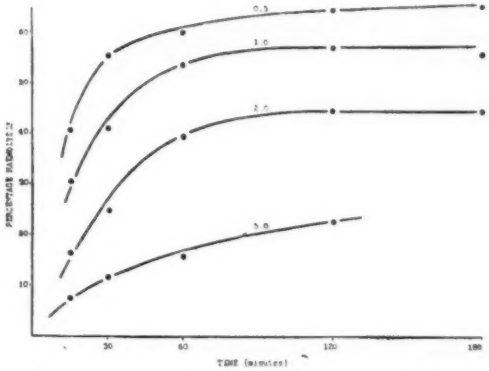

Fig. 4. Course of haemolysis according to concentration of red cells. From same experiment as Fig. 3, pefeentage by volume of packed fowl cells shown on curves. Ordinates percentage haemolysis.

4. The final level depends on the receptor status of the red cells, damage to receptors by virus on the receptor destroying enzyme RDE of *Vibrio cholerae* diminishing the level in proportion to the degree of damage. This feature which dominates the whole process of haemolysis is considered in the next section.

TABLE 3.

Action of allantoic fluid virus on human and fowl cells in three hours at 37° C.

Virus	Human cells				Fowl cells			
	0·5%	1·0%	2·0%	5·0%	0·5%	1·0%	2·0%	5·0%
1:5	0·14	0·20	0·28	0·53	0·21	0·66	1·47	3·38
1:10	0·12	0·16	0·22	0·33	0·17	0·39	0·97	2·16
1:20	0·11	0·11	0·15	0·23	0·12	0·22	0·46	0·87

The final level of haemolysis is shown as gm./l. of HbO_2.

6. *Inhibition of Haemolysis by Receptor Destruction.*

The most striking feature of NDV haemolysis which immediately differentiates it from every other type of haemolysis (except haemolysis by mumps virus) is the complete resistance of cells from which virus receptors have been removed.

The following experiment with human cells is typical. Human cells at a concentration of 2 p.c. in buffered calcium saline were treated for 1 hour at 37° C. with serial dilutions of RDE (crude extract). Others were treated at room temperature for 3 hours with the higher concentrations of RDE. Another set was treated with the viruses Melb. A, LEE B and SW 15 for 3 hours at 37° C. with undiluted allantoic fluid. The cells treated with RDE were washed first with saline containing 1 p.c. sodium citrate to inhibit further action of the enzyme and then with normal saline. The cells treated with viruses were unstable and were stabilized by suitable low concentrations of homologous ferret immune sera followed by a further washing with saline. All treated cells with normal human cells as control were tested with the gradient series of viruses and a 1 p.c. suspension in phosphate saline treated with 1:10 MPV (approximately 125 agglutinating doses) for 90 minutes at 37° C. The degree of haemolysis was measured from the amount of haemoglobin in the supernatant fluid.

TABLE 4.

Relation of receptor removal to haemolysis by NDV.

Treatment of human cells	Virus gradient									Haemolysis gm./l.
	N.D.	MEL	WS	MU	LEE	BEL	SW	MIL B	N.S.	
RDE 1:10 37° C.	—	—	—	—	—	—	—	++	—	—
RDE 1:20 37° C.	—	—	—	—	—	—	—	++	—	—
RDE 1:40 37° C.	—	—	—	—	—	+	+	++	—	—
RDE 1:80 37° C.	—	—	—	—	++	++	++	++	—	—
RDE 1:160 37° C.	—	—	±	±	++	++	++	++	—	—
RDE 1:5 RT	—	—	—	—	—	—	—	—	—	—(0·07)
RDE 1:10 RT	—	—	—	—	—	—	—	—	—	—(0·08)
MEL A	—	±	±	—	++	++	++	++	—	+ 0·33
LEE B	—	—	—	—	++	—	++	++	—	tr (0·11)
SW 15	—	—	—	—	—	—	—	++	—	—
Normal cells	++	++	++	++	++	++	++	++	—	++1·99

TABLE 5.

Character of cells after preliminary treatment with NDV

	Virus	Temperature	Time	Diluent	Stability	I.F.S.
A	MPV/20	37° C.	30′	Ca BS	—	++
B	AF/10	37° C.	30′	Ca BS	—	++
C	AF/10	37° C.	10′	P.S.	+	++
D	MPV/20	17° C.	2 hr.	P.S.	++	—
E	AF/10	17° C.	2 hr.	P.S.	++	—
F	Nil	—	—	P.S.	—	—

MPV Methanol-precipitated virus. AF Allantoic fluid virus. Ca BS Buffered calcium saline. P.S. Phosphate saline.

The results show clearly that when reactivity with MEL is lost as judged by our standard test, haemolysis by NDV fails to occur.

The influence of prior treatment with NDV or subsequent haemolysis by the same virus is of particular interest. In order to avoid gross haemolysis the first treatment may be made

in the presence of Ca at 37° C., at room temperature or by the use of a short exposure only at 37° C. with allantoic fluid virus. Table 5 shows the treatment applied and the characteristics of the cells.

The receptor changes were not measured in this experiment but the results from similar treatment have been consistent in indicating that cells A, B and C would be resistant to NDV and MEL while cells D and E when stabilized by immune serum would not be agglutinated by NDV but would show some agglutination by MEL.

The washed cells were then added to give a final concentration of 1 p.c. in phosphate saline containing 1:10 of MPV, previously warmed to 37° C. Samples were taken at appropriate intervals for determination of haemoglobin in the supernatant fluid with the results shown in Fig. 5.

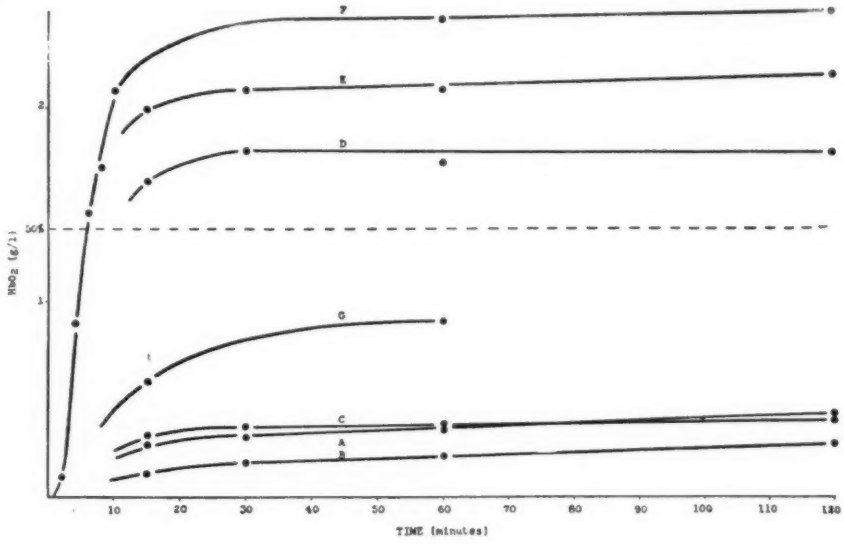

Fig. 5. Haemolysis by MPV of cells previously treated with NDV under various conditions.

A, B, C cells treated with virus at 37° C., D, E cells treated with virus at 17° C., F normal fowl cells. For details see Table 6.

The chief interest of the results is the demonstration that the action of virus at room temperature has a small but definite effect in reducing the level to which haemolysis extends and that even pretreatment at 37° C. does not quite remove the susceptibility to haemolysis.

Included in the same experiment were cells which had been treated with MPV heated 52°, 54° and 56° C. for an hour in phosphate saline at 37° C. The results with cells treated with 54° C. virus are included as line G on the Figure. The value for these cells at 15 minutes represents 21 p.c. haemolysis, the other values at 15 minutes were: Control cells >90 p.c., cells treated with 52° C. virus, 12 p.c., cells treated with 56° C. virus, 45 p.c. haemolysis.

7. *The Initial Stages of Haemolysis.*

Detailed investigation of the first portion of the curve of haemolysis has given results varying with the type of preparation used. With the best MPV preparations there is a lag period of 1–2 minutes only followed by rapid haemolysis. Preparation Batch 10 which in other respects also was intermediate in character notably in giving a higher final haemolysis level with fowl than with human cells, showed a period of slow haemolysis lasting 8–10 minutes with a subsequent rapid phase. Untreated allantoic fluids varied considerably from batch to batch but in general haemolysis of fowl cells began slowly and increased steadily for an hour or more. With human cells the initial phase was more rapid with only a slow subsequent increase in haemolysis. In most instances the curves for haemolysis of human and fowl cells crossed the end value being always higher for fowl cells. Eluates prepared by adsorbing virus to fowl cells at 0° C. and after washing with cold saline allowing elution to proceed at room temperature for 18–20 hours, behaved similarly.

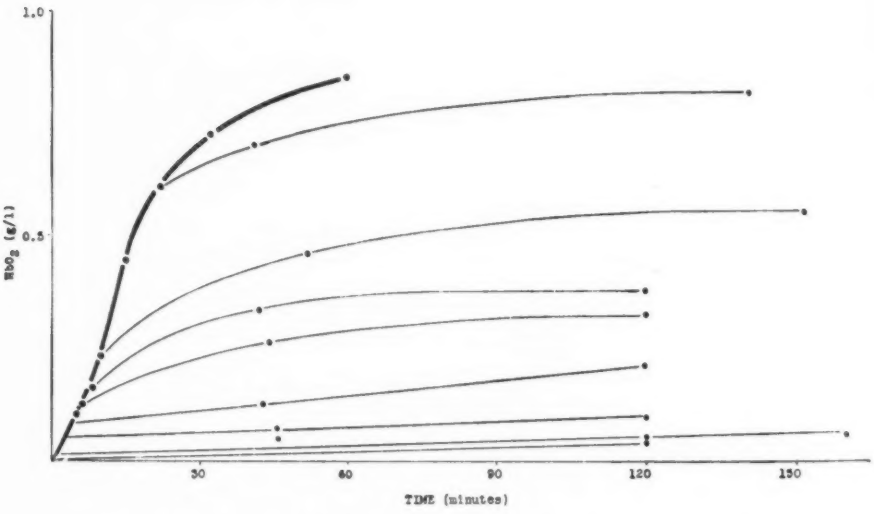

Fig. 6. Initiation of haemolysis at 37° C. with subsequent haemolysis at 17° C. Samples removed and chilled to 17° C. at times shown on the main curve.

The impression gained over an extensive series of experiments was that the population of virus particles in any given preparation is always heterogeneous in respect to their haemolytic activity and that the major difference is in the lag period which elapses between adsorption of the virus particle to the cell and the occurrence of haemolysis. This heterogeneity naturally makes it extremely difficult to devise experiments in regard to the actual kinetics of the haemolytic process.

The MPV preparation Batch 10 was used in two concordant experiments to demonstrate that the process initiated by virus at 37° C. could proceed subsequently to haemolysis under conditions which would prevent the initiation of haemolysis.

A series of tubes was set up at 37° C., each containing the same mixture of MPV Batch 10 at a final dilution of 1:10 and 1 p.c. human cells in phosphate saline. At intervals a tube was removed, chilled rapidly in ice water to 17° C. and maintained at that temperature. Samples were taken at the time of removal and at succeeding intervals for estimation of free haemoglobin. The course of haemolysis with the mixture at 37° C. throughout was also determined. A trace of haemolysis (0·04 gm./l. HbO_2 at 120 mins.) was shown in the mixture held at 17° C. throughout. The values for the other mixtures were corrected by subtracting 0·04 from the figures obtained at 2 hours and 0·02 from those at 40–50 minutes. The results illustrated graphically in Fig. 6 show clearly that although haemolysis cannot be initiated at 17° C., it will proceed at that temperature after initiation at 37° C. The initiating reaction proceeds fairly slowly since the maximum extent of subsequent haemolysis results in the sample taken after 10 minutes' contact at 37° C. The suggestion is strong that in this instance the pacemaker for haemolysis is the initiating reaction rather than the subsequent liberation of haemoglobin.

It is not practicable to carry out this type of experiment with highly active MPV which does not show an appreciable lag period. The following experiment indicates, however, that with such virus almost the whole of the initiating reaction is complete long before haemolysis reaches the final level. A series of tubes containing MPV Batch 26 at a final dilution of 1:33 with 1 p.c. human cells was set up at 37° C. At intervals individual tubes were chilled and centrifuged. The supernatant fluid was removed for haemoglobin estimation and the cell deposit resuspended in a volume of saline equal to the original. These tubes were left 4 hours at 18° C., marked haemolysis occurred and after centrifugation the haemoglobin was estimated in the supernatant fluid. The results shown in Table 5 indicate that even after 7 minutes at 37° C. the cells are capable of completing haemolysis at room temperature. The possibility should be borne in mind that some of the haemolysis observed may be a result of the trauma of centrifugation on damaged cells.

TABLE 6.

Completion of haemolysis at 18° C. after initiation at 37° C.

Time at 37° C.	Haemoglobin content		
	First supernatant	After 4 hours at 18° C.	Total
7′	0·72	1·21	1·93
15′	1·05	0·88	1·93
30′	1·48	0·58	2·06
110′	1·93		

100 p.c. haemolysis = 2·18 gm./l.

The conclusion from these experiments that with active MPV the initiation of haemolysis is virtually completed at an early stage is further substantiated by experiments with RDE reported in the next section.

8. *Analysis of Kinetics of Haemolysis by Use of RDE.*

The vibrio enzyme RDE has an extremely rapid action in rendering red cells insusceptible to the action of NDV. This makes it possible to analyse a little further the process of haemolysis by the virus. With purified RDE (Ada *et al.*) relatively high concentrations can be obtained in water clear solution so that no correction for colour is necessary. A convenient concentration for work with the usual range of virus and cell concentrations is a final dilution of 200 units.

The speed of action of the enzyme can be shown from the following experiment in which MPV Batch 26 was used at a final dilution of 1:20 with 1 p.c. human cells. Except for the control which received an equivalent volume of saline, cell mixtures contained in addition 200 units of RDE. The order of mixing the reagents is shown in the Table, and the degree of haemolysis was read after 2 hours at 37° C. All reagents were brought to 37° C. before mixing.

It will be seen that after treatment of the cells for one minute with RDE haemolysis is reduced to about 7 p.c. of the control. In other words when this concentration of RDE is added to a reacting mixture of virus and cells the possibility of significant new haemolysis being initiated ceases within 1 or 2 minutes.

TABLE 7.

Action of RDE in inhibiting haemolysis by MPV.

Order of mixing		Haemolysis after 2 hours gm./l. HbO_2
Cells	virus — 10″ — RDE	1·197
Cells	RDE — 1′ — virus	0·120
Cells	RDE — 2′ — virus	0·072
Cells	RDE — 5′ — virus	0·060
Cells	virus only	1·854 (85 p.c. haemolysis)

In the experiment shown in Fig. 7 two similar mixtures of 1 p.c. human cells and MPV Batch 26 1:40 were used to one of which 200 units of RDE were added one minute after the virus and cells had been mixed. All reagents were warmed to 37° C. and the tubes maintained at that temperature throughout, samples were taken at intervals, chilled and the haemoglobin estimated as usual.

It will be seen that the general shape of the curve is hardly altered from the normal but the final level is reduced from 2·085 to 1·39 gm./l. HbO_2. This can be interpreted as indicating that about 67 p.c. of the cells which will eventually haemolyse have undergone the initiating reaction with 2 minutes of mixing cells and virus, and that the lower (RDE) curve is essentially a measure of the process of haemolysis in cells on which the "haemolytic" reaction between virus and receptor had taken place within 2 minutes.

This result suggested that if virus and RDE were first mixed and cells exposed at 37° C. to the simultaneous action of the two reagents, the resulting haemolysis would be equivalent to that shown by a lower concentration of virus; the higher the ratio of RDE to virus the greater the reduction in haemolytic activity. In one experiment with Batch 26 virus and 1 p.c. human cells the addition of 200, 100 and 50 units to each tube of a series, gave curves which would be closely similar to those produced by the same virus at 1/11, 1/7 and 1/3 the actual concentration, respectively.

With actively haemolytic MPV it appears that the part played by the virus in initiating haemolysis is rapidly completed and that the observed course of haemolysis is largely a measure of secondary processes taking place in the cell which require neither the presence of surface receptors nor a temperature above 26° C. in order to take place.

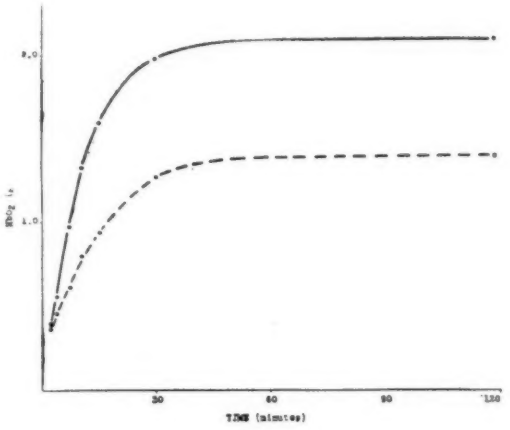

Fig. 7. Influence of addition of RDE one minute after mixing cells and virus. Solid line—control. Broken line—added RDE.

9. *The Effect of Ca Ion Concentration on Haemolysis by NDV.*

The influence of Ca ion concentration on haemolysis by NDV varies strikingly with the type of virus preparation and the species of red cell used. The partial inhibition of haemolysis in undiluted allantoic fluid virus as compared with 1 : 5 or 1 : 10 dilutions can be removed by Ca-deionizing agents and appears to be due simply to Ca. In general unprecipitated virus is less active in the presence of Ca and haemolysis of fowl cells is more strongly inhibited than haemolysis of human cells.

With MPV most preparations show a considerable reduction in haemolytic activity in saline containing 0·1 p.c. $CaCl_2$ as compared with phosphate saline on fowl cells. With some preparations tested on human cells no effect whatever was evident.

As an example of the difference between fowl and human cells the following rough experiment with MPV Batch 10 may be cited. A series of $CaCl_2$ dilutions in physiological saline was prepared ranging from 0·1 p.c. downwards. To 0·5 ml. of each dilution 0·25 ml. of MPV (haemagglutinin titre 400) was added and then 1 drop of 20 p.c. cells (fowl or human). After 1 hour at 37° C. the degree of haemolysis was as shown in Table 8.

TABLE 8.

Inhibition of haemolysis by Ca.

Cells	Concentration of $CaCl_2$% in normal saline								
	0·067	0·05	0·04	0·033	0·027	0·02	0·013	0·007	0
Human	15	15	17	20	25	30	40	50	70
Fowl	—	—	—	5	6	7	10	20	60

Approximate p.c. haemolysis at 1 hour shown.

Where a preparation is almost non-haemolytic in the presence of 0·1 p.c. $CaCl_2$ it can be shown that the initiating reaction leading to haemolysis nevertheless takes place. In the experiment whose results are shown in Fig. 8 samples of mixtures reacting in 0·1 p.c. Ca saline at 37° C. were at the intervals shown brought up to standard volume by the addition of one fifth the volume of 2 p.c. citrate saline and chilled to 17° C. The course of haemolysis was then followed over 2 hours.

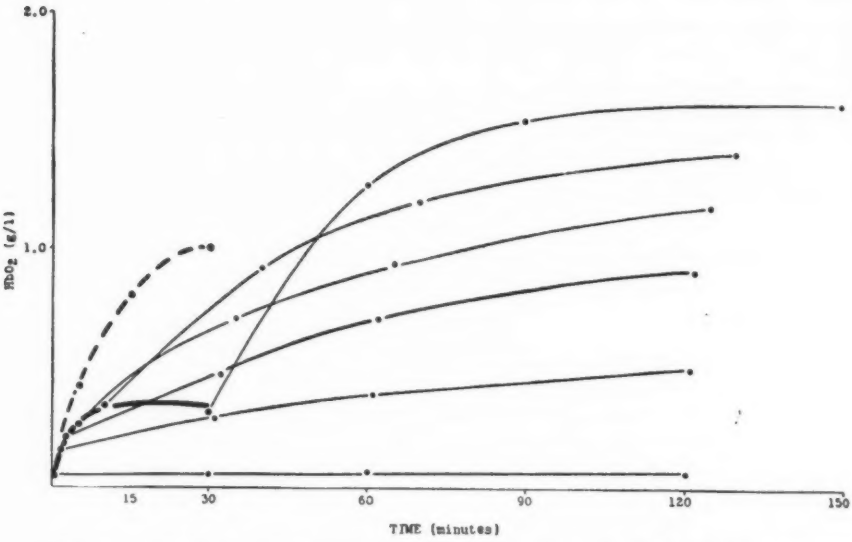

Fig. 8. Heavy solid line—Course of haemolysis at 37° C. in saline containing 0·1 p.c. $CaCl_2$. Fine lines show course at 17° C. in samples taken at times indicated and brought up to standard volume with citrate. Heavy broken line—Haemolysis at 37° C. in control Ca + citrate mixture.

The results are of the same general character as those shown in Fig. 6 indicating that up to 30 minutes at least many contacts capable of initiating haemolysis had been made which would produce haemolysis only after removal of Ca.

10. *Neutralization of NDV Haemolysis by Immune Serum.*

In view of the appearance of the paper by Kilham (1949) in which the neutralization of NDV haemolysis by specific immune serum is reported, it is not necessary to give detailed experiments to confirm his finding. Using immune sera prepared by Anderson (1947) and stored in the lyophilized state haemolysis was found to be inhibited to titres corresponding very closely with the antihaemagglutinin titre of the sera.

In a previous paper Burnet (1949) it was shown that ovomucin treated with relatively large amounts of periodate was an inhibitor of haemagglutination by active NDV and of none of the other viruses of the group. Solutions of periodate-treated ovomucin had an equivalent action in preventing haemolysis by NDV. Untreated ovomucin has no such effect. A full account of work on the interaction of NDV and ovomucin will be published later.

11. *The Coexistence of Different Properties of NDV on Firmly Fixed Virus Particles.*

In the preceding paper it has been shown that the conditions under which irreversible fixation to red cells takes place are the same as those necessary for haemolysis. It was therefore of interest to examine whether virus fixed to cells or stromata was capable of haemolytic activity. For obvious reasons it was unsatisfactory to attempt to use intact erythrocytes and the experiments have been made in two directions first by the study of stromata produced by the haemolytic action of MPV and second by treatment of saponin stromata with virus preparations. For ease of handling, fowl cell stromata were used in both types of experiment.

If fowl cells are treated with high concentrations of MPV a high proportion of stromata can be obtained. By washing with saline and at each centrifugation discarding as much as possible of the unaltered cells it is possible to obtain uniform material light brownish pink in colour containing no intact red cells. This liberates traces of haemoglobin with further handling and control tubes to allow for this are required in any haemolytic experiments.

Almost pure white preparations can be obtained by saponin haemolysis and repeated washing. These stromata, however, have a persistent slight haemolytic effect due to residual saponin and in order to avoid this the routine procedure has been to test in the presence of 1:100 saline dilution of fowl sera both normal and immune to NDV. Complete absence of haemolysis in the presence of immune serum will then indicate that any haemolysis in the presence of normal serum is due to NDV.

All experiments have shown that stromata prepared by MPV haemolysis, when treated with RDE and thoroughly washed are still capable of inducing haemolysis of normal cells.

TABLE 9.

Haemolytic action of NDV—stromata on human cells.

	HbO_2 gm./l. 3 hours 37° C.
NDV—Stromata + H.C.	0·310
Stromata alone	0·108
H.C. alone	0·025

H.C. = Human cells.

In the experiment shown in Table 9 fowl cells (0·25 ml.) were treated with MPV B23 (2 ml.) for 30 minutes at 37° C. The supernatant fluid was then replaced by saline containing approximately 200 units of RDE and held for another 30 minutes at 37° C. The stromata were then washed first with 1 p.c. citrate saline and then twice with normal saline, the unlysed cells being discarded in the process. The stromata were suspended in 5 ml. saline and haemolytic tests carried out with 1 ml. of stromata and 1 ml. of 5 p.c. human cells made up to 5 ml. with phosphate saline. Samples were taken at hourly intervals but only the 3 hour results are shown. No significant action was shown on RDE treated cells and none in the presence of anti-NDV serum.

Any type of NDV can be adsorbed to saponin stromata at 37° C. giving essentially the same phenomena as those observed with intact cells. All experiments have shown that the ASC titre of the stromata against normal fowl cells is of the same order as would be observed with similarly treated intact cells and that it is not diminished by RDE treatment.

In the following experiment 1 ml. lots of a stromata suspension approximately equivalent to 5 p.c. cell suspension were treated with two MPV preparations 23D and 24A and a "cold eluate" from unprecipitated virus. The haemagglutinin titres were respectively 400, 1,600 and 700. After 30 minutes at 37° C., RDE was added and the stromata subsequently washed with citrate saline and two changes of normal saline. The stromata made up to 1 ml. were then tested for ASC and for haemolytic action on human red cells.

TABLE 10.

Reactions of saponin stromata treated with NDV and RDE.

Stromata	Haemolysis 3 hours		A S C titre
	N.F.S.	I.F.S.	
MPV 230	0·206	<0·02	10
MPV 24A	0·700	0·054	48
Cold eluate	0·156	0·028	6

In previous work Anderson (1947) showed that the NDV particles firmly attached to red cells had all the characteristic attributes of the virus. The present experiments have incidentally confirmed most of the points made by Anderson and in addition have shown that the haemolytic activity of the particles if present initially is retained. It is not possible to decide whether the virus attached to stromata is more or less active as a haemolysin than full virus, since the actual amount on the stromata is not known and it is impossible to allow for the influence of the carrier in any quantitative fashion. Table 10 has however, been chosen to exemplify the reactions of saponin stromata because the preparation 24A which had a very low grade haemolytic activity as prepared, appears to have developed a considerably increased action after fixation on stromata and subsequent washing.

DISCUSSION.

There can be no doubt that the haemolytic agent concerned in these phenomena is the virus particle itself. This conclusion is based on the fact that the haemolytic agent can be adsorbed and eluted from red cells, is inactivated by immune serum and periodate-treated ovomucin, in part can be irreversibly fixed to washed stromata. This conclusion has already been reached for mumps virus by Morgan, Enders and Wagley (1948), by Kilham (1949) for NDV and by Clarke and Fox (1948) for haemolysis by the rickettsiae of murine and epidemic typhus.

Any attempt to go more deeply into the processes concerned in the surface interactions between viruses and cells comes up immediately against our ignorance of the fine structure of the surfaces concerned. Both the "receptor destroying" activity of the whole group of viruses and the haemolytic action of mumps virus and NDV are clearly enzymic in character. The reactions however are of a different order of complexity from the more familiar reactions

between soluble enzymes and chemically defined substrates and precise conclusions are not to be expected. Nevertheless, there is much to be said for the view that the great majority of biologically important enzymic actions—those taking place within the cell—are interactions between components held at surfaces. It may be that an accessible model of such enzymic actions might be developed from the virus-cell reactions of the present type. This is perhaps the main justification for an attempt to discuss the characteristics of the reaction in considerable detail.

We have been restricted to a single strain of Newcastle disease (of Australian origin) owing to necessities of quarantine. Although this strain has been shown to be serologically identical with European and by implication with American strains, and has all the recognized qualities of the virus, its behaviour will almost certainly differ in detail from that of other strains. Kilham's (1949) description suggests a more actively haemolytic strain than ours, and other differences might well be expected from the strains producing mild pneumo-encephalitis in fowls. Without a comparative examination of a wide series of strains it would be unjustifiable to claim that the reactions described are characteristic of the species. However, the interesting resemblances between mumps and Newcastle disease viruses first indicated in 1945 (Burnet, 1945) and now strengthened by the demonstration of the haemolytic power of both viruses, offers justification for regarding the phenomena of virus haemolysis as shown by Newcastle disease virus as of potential importance for the general problem of virus action.

The outstanding characteristic of NDV haemolysis is the necessity for receptors to be present on the cells. Rabbit cells which are not agglutinated are not haemolysed. Cells treated with RDE are quite unaffected. The cessation of haemolysis at a certain level depends on the destruction of receptors by concomitant non-haemolytic action of the virus. Whenever a haemolytic test is set up several types of virus-receptor action occur, certainly three and probably four. These reactions "compete" for the available receptors and haemolysis may be taken as a measure of the proportion of cells in which a haemolytic process is successfully initiated before all available receptors have been removed or blocked by the other actions.

The four types of action which may be mediated by the cell receptors are (1) the standard process of adsorption, enzymic action and release of the virus when the receptor has been modified to the full extent possible by the virus in question. (1a) Incomplete phases of the same process such as are seen for instance with virus heated to 55° C. The virus is adsorbed firmly enough to allow visible agglutination of cells but the enzymic action is obstructed in one way or another. Cells to which virus is so attached are spontaneously agglutinable and it is characteristic that small amounts of specific immune serum stabilize such cells, in all probability by removing the virus from the surface. (2) Irreversible union of virus particles to

cell receptors. This can occur only above a critical temperature whose level depends on the type of cell being studied. The attached virus particles cannot be removed by RDE or by immune serum. Since they remain firmly attached immune serum agglutinates cells to which virus is adsorbed in this fashion.

The three foregoing reactions can all be demonstrated in the complete absence of haemolysis so that a fourth mode of virus-receptor interaction (type 3) must be postulated to account for haemolytic action.

All four reactions fail to appear (A) if the cell receptors are destroyed by RDE or a suitably active influenza virus; (B) if an agent capable of combining firmly with the virus particle is present—immune serum or periodate-treated ovomucin. No evidence has been obtained to suggest that there is any significant heterogeneity amongst red cells in regard to the nature of their receptors. Current work by Ada and Stone (1950) in this laboratory on changes in electrophoretic mobility produced by viruses and RDE speaks strongly in favour of their uniformity. The whole trend of the results points to the virus as the factor determining which type of reaction occurs.

Our working hypothesis is that on the surface of the virus particle there are active patches, atomic configurations with a specific complementary relationship to patterns presented by mucopolysaccharide molecules or aggregates on the cell surface. As long as the active patches are present they will be adsorbed to accessible (mucopolysaccharide) receptors. In their most typical state adsorption is followed by enzyme action which disrupts the receptor (substrate) pattern so that the latter no longer binds the active patch of the virus. At the present time it seems to be immaterial whether we equate active patch with enzyme or regard the enzymic action as the responsibility of a portion only of the adsorptive area of the virus. Throughout all our work in this general field there is a very detailed correspondence between the adsorptive capacities of a virus in its "passive" rôle e.g. as an indicator of haemagglutination inhibitors, with its range of enzymic action in the active state. The simplest view is probably to regard the active patch as essentially a mucolytic enzyme having the type of complementary steric relation to the substrate portion of the mucin, that is regarded by Gottschalk (1950) as general for enzyme-substrate relationships. The disposition of the enzyme groupings proper in relation to other surface components of the virus must have an important bearing on the ability or otherwise of enzyme and substrate—both being as they are embedded in complex surfaces—to make effective adsorptive union. At the present time it seems to be impossible to go farther than to postulate a series of relatively minor changes in the configurations that make up the active patches of the virus particle. The primary adsorptive attraction of the two patterns (potential

enzyme and potential substrate) persists in all the situations capable of presenting demonstrable effects, but the result of any particular adsorption will be determined by associated conditions. In the last analysis an enzyme is merely a particular electronic configuration which under appropriate conditions can induce the rearrangement of another electronic configuration (the substrate). An enzyme can be adsorbed to a configuration which it cannot break down with resulting competitive inhibition of enzymic action on the normal substrate. It appears equally reasonable that minor modifications of the enzyme pattern may leave its adsorptive qualities almost intact but eliminate the possibility of enzymic action.

To transfer these ideas to the virus particle of Newcastle disease we assume that a number of variations are possible in the overall pattern of the active patches which are distributed over the virus surface. These variants may be labelled 1, 2 and 3 according to the actions mentioned above. In the virus particles as harvested from the chick embryo some may have active groups of all four types but many probably lack 1a and 3. When virus is heated to 54° C. 2 and 3 disappear as such, 1 diminishes, probably being largely converted into 1a, i.e. to groups conserving adsorptive power but lacking actual enzymic activity. When virus is precipitated by methanol type 3 patches become much more evident perhaps as a result of the removal of some absorbed inhibitor, perhaps by actual conversion of other types of patch.

It does not seem possible to decide whether patches of type 2 and 3 react with receptors at the lower temperature according to modes 1 and 1a or fail to react at all. No explanation whatever can be offered as to why type 3 enzyme-receptor union results in haemolysis. It is tempting to believe that types 2 and 3 union are essentially similar, that a basically different type of mutual orientation of enzyme and substrate from type 1 and 1a occurs and that two sequels are possible. Either a firm irreversible chemical union develops (type 2) or an essentially different type of releasing enzymic action which is the initiating phase of a process leading to haemolysis. It has not been possible to prove that after acting haemolytically a virus particle is released nor, in view of the fact that it has been proved that a virus particle irreversibly fixed to a cell surface may manifest with its free surface any one of the 4 types of action, is it easy to see how it ever could be proved unless, by some means virus having only potentially haemolytic active patches could be prepared.

The actual course and final level of haemolysis are determined by the number of type 3 contacts which under the existing circumstances can be made before all the available points of union have been removed either by the virus itself or in certain experiments by the added RDE. Receptors can be rendered unavailable to type 3 action either by enzymic destruction (virus or RDE) or by being blocked by firmly held virus particles.

The haemolytic process that follows the initiating reaction is one whose speed seems to vary widely for individual erythrocytes. There is never complete liberation of haemoglobin, the washed stromata from fowl cells lysed by NDV are light brownish pink in colour contrasting with the almost pure white stromata obtained by saponin haemolysis. If the cells from a mixture showing about 75 p.c. of its maximal degree of haemolysis are transferred to a capillary tube and centrifuged, three cell layers will be seen, a bottom layer of firmly packed deep red unhaemolysed cells, an intermediate bright red layer with a graded transition to the upper level of pale brown stromata. If the intermediate level is separated by breaking the capillary at appropriate points, and emulsified in saline haemolysis rapidly follows. One does not, however, gain the impression that there are all degrees of haemolysis. The main parts of the cell column in such experiments are uniformly coloured stromata or apparently normal red cells, the intermediate unstable zone appearing to represent merely a short-lasting transitional phase.

The logarithmic relation of final levels of haemolysis to virus concentration would be expected if we had two competing enzyme reactions 1 and 3 in which the turnover number for 1 was higher than that for 3. If as seems likely the turnover number is much higher a very large proportion of the initiating reactions will be derived from *first* type 3 contacts and it is conceivable that some proportion of such contacts may initiate haemolysis without releasing the virus particle.

When virus adsorbed to cells in the cold is allowed to elute at 17° C. the eluate has approximately the same haemolytic activity as the original virus. If the elution is at 37° C there is a significant diminution in haemolytic power. This does not necessarily mean more than that a relatively higher proportion of virus particles capable of being fixed readily to cell receptors has haemolytic power than is the case with virus particles not readily fixed. There is no doubt that Anderson (1947) was correct in regarding NDV populations as heterogeneous but instead of ascribing this heterogeneity to different levels of aggregation we would regard it as resulting from the varied development and activity of active patches (potentially enzymic in character) on different virus particles. Some evidence both of morphological heterogeneity and lability is obtainable from electron micrographs but little or none of different degrees of aggregation.

A whole series of problems raised by the phenomena of NDV haemolysis have had to be left untouched in this discussion. One of the most interesting is the greater haemolytic effect of allantoic fluid on fowl cells while MPV is much more active on human cells. Work on the change in the virus produced by methanol precipitation is being continued but at the present time there are practically no clues as to its nature.

SUMMARY.

Newcastle disease virus is haemolytic for several species of mammalian and avian erythrocytes.

Allantoic fluid virus is only weakly haemolytic and is relatively more haemolytic when diluted than when used undiluted.

Virus precipitated from allantoic fluid with methanol in the cold (MPV) and dialysed free of methanol is much more actively haemolytic than the original.

Haemolytic activity is lost by heating at 54° C. for 30 minutes a treatment which leaves intact the haemagglutinating and inhibitor destroying activities of the virus.

At temperatures below 24° C. (human cells) or 28° C. (fowl cells) the speed of haemolysis falls very rapidly and at 18° C. or below it is inappreciable.

With an actively haemolytic virus and a high virus/cell ratio haemolysis may be complete but in general haemolysis proceeds only to a certain level and then virtually ceases.

The final level is determined by the relative concentrations of virus and cells, the type of virus and the degree to which cell receptors have been modified by previous treatment.

Cells from which receptors are removed by the action of RDE or other viruses are completely resistant to haemolysis, provided the receptor destruction has proceeded to a point corresponding to loss of agglutinability by strain MEL.

The final level of haemolysis can be reduced to any desired value by appropriate preliminary treatment of the cells with NDV under conditions which do not produce haemolysis.

The initiation of haemolysis can take place only at a temperature above a critical point but once initiated haemolysis will proceed at a lower temperature. The speed with which the initiating reaction occurs is the main factor determining the shape of the haemolytic curve.

Calcium has an inhibiting effect on haemolysis much more evident with fowl cells than with human cells and with allantoic fluid virus than with MPV.

Haemolysis is inhibited by immune serum to titres corresponding closely to the anti-haemagglutinating titre of the serum.

Virus firmly attached to stromata (saponin lysed fowl cells) can haemolyse added normal red cells.

The phenomena are discussed on a basis of the hypothesis that there are several types of active patch on the virus particle surface.

REFERENCES.

Ada, G. L. and Stone, J. D. (1950): Nature,
Ada, G. L., French, E. L., Curtain, C. C. and Laver, W. G. (in preparation).
Anderson, S. G. (1947): Austral. J. exp. Biol., 25, p. 163.
Burnet, F. M. (1945): Austral. J. Sci., 8, p. 81.
Burnet, F. M. (1949): Austral. J. exp. Biol., 27, p. 361.
Burnet, F. M., McCrea, J. F. and Stone, J. D. (1946): Brit. J. exp. Path., 27, p. 228.
Burnet, F. M. and Stone, J. D. (1947): Austral. J. exp. Biol., 25, p. 227.
Clarke, D. H. and Fox, J. P. (1948): J. exp. Med., 88, p. 25.
Cox, H. R., van der Scheer, J., Aiston, S. and Bohnel, E. (1947): J. Immunol., 56, p. 149.
Gottschalk, A. (1950): Advances in Carbohydrate Chemistry, Academic Press, New York, Vol. 5.
Holden, H. F. (1946): Austral. J. Inst. Technol., p. 121.
Kilham, L. (1949): Proc. Soc. exp. Biol., 71, p. 63.
Morgan, H. R., Enders, J. F. and Wagley, P. F. (1948): J. exp. Med., 88, p. 503.
Stone, J. D. (1947): Austral. J. exp. Biol., 25, p. 137.

GLYCOLYSIS IN OYSTER MUSCLE

by GEORGE FREDERICK HUMPHREY[1]

(From the Department of Biochemistry, University of Sydney).

(Accepted for publication 11th October, 1949.)

Previous investigation on muscle glycolysis in this species (Humphrey, 1944) has shown that glycogen is broken down in the presence of added phosphate, K, Mg and diphosphopyridinenucleotide (DPN) to give a mixture of pyruvic and lactic acids. In that investigation the whole adductor muscle was used to prepare aqueous extracts.

Principally because of the work of Marceau (1909) but also through analogy with other species, it is possible to assign different functions to the two morphological parts of which the adductor muscle is composed. The nacreous (smooth, slow, white) part is supposed to function by keeping the width of shell opening at any value set by the activity of the vitreous (striated, quick, grey) part. In other words, the vitreous portion can be regarded as being used for making shell movements while the nacreous portion simply maintains the state determined by the activity of the vitreous part. Therefore the nacreous part is largely responsible for the prolonged shell closure which occurs when the animal is kept out of water; under cool conditions, this state of closure can be maintained for 2–3 weeks. Thus one part of the muscle is called upon to function steadily over long periods while the other is used for sudden activity.

The importance of adenosinetriphosphate (ATP) in energy transfer is now well known and the concentration and breakdown of this compound in the two parts of the muscle has been studied (Humphrey, 1949a). The relationship between ATP and glycolysis makes it necessary to continue the study of the metabolism of the adductor muscle by examining the glycolytic patterns exhibited by the nacreous and vitreous tissue.

EXPERIMENTAL.

Material.

The animals and chemicals were as previously used (Humphrey, 1950).

Methods.

In general the techniques used were those of previous papers (Humphrey, 1941, 1944, 1946 and 1950).

[1] This investigation was supported by a grant from the Research Committee, University of Sydney.

Results.

Intensity of Glycolysis.

A comparison between the two parts of the muscle with regard to the production of pyruvic and lactic acids is set out in Table 1. It can be seen that both with extracts and homogenates the vitreous tissue is much more active than the nacreous. The amount of glycolysis varied with the phosphate concentration; this was best shown with extracts, where reduction of the phosphate level to 0·001 M brought the amount of acid production to about a tenth of the level with 0·03 M phosphate. At these lower phosphate concentrations the ratio of pyruvate to lactate formed by the vitreous part dropped to about 0·5 or less. If the experiments with homogenates were carried out in the presence of air or O_2, there was a slight drop in the amount of lactic acid but a slight increase in the amount of pyruvic acid.

TABLE 1.

Amount of acid production.

Extracts or homogenates incubated 150 minutes under N_2 in the presence of 0·005 M $MgCl_2$, 0·001 M DPN, 0·002 M ATP, 1 p.c. glycogen and 0·03 M phosphate. The Q values are calculated on the basis of mg. N in the extract or homogenate.

	Extract		Homogenate	
	Nacreous	Vitreous	Nacreous	Vitreous
Q pyruvate	0·4	69	0·08	0·8
Q lactate	3·8	46	1·2	3·2

Manometric estimation of glycolysis showed that there was close agreement between chemical and manometric values when extracts of vitreous tissue were used but that the nacreous part gave large discrepancies, only a fraction of the acid expected on manometric grounds being found chemically (Table 2).

TABLE 2.

Manometric acid production.

Extract incubated 160 minutes under 5 p.c. CO_2–95 p.c. N_2 in Warburg flasks in the presence of 0·005 M $MgCl_2$, 0·001 M DPN, 0·002 M ATP, 1 p.c. glycogen, 0·05 M phosphate and $NaHCO_3$ to give a final pH of 7·3.

	Nacreous tissue	Vitreous tissue
μg. acid (manometric)	172	260
μg. acid (chemical)	38	234

The total CO_2 of the system was estimated by the method of Krebs and Johnson (1937) in experiments where the gas phase was 1 p.c. CO_2—99 p.c. N_2 and the $NaHCO_3$ concentration lowered to keep the pH at 7·3; there was no *de novo* production of CO_2 and therefore the formation of "manometric acid" must be due to acid production. No steam-volatile acids could be detected by the method of Friedemann (1938); the results given under the section on *Phosphate Esterification* indicate that this extra acid production is due to splitting of P_7 from ATP by phosphatases which are more active in the nacreous than the vitreous tissue (Humphrey, 1949 a).

Substrates.

Compounds other than glycogen were tried as possible substrates for glycolysis (Table 3).

TABLE 3.

Effect of substrates on glycolysis.

Extracts or homogenates were incubated 120 minutes under N_2 in the presence of 0·005 M $MgCl_2$, 0·001 M DPN, 0·002 M ATP, 0·05 M phosphate and the substrates as shown.

	Pyruvic acid	Lactic acid	Pyruvic acid	Lactic acid
	Nacreous extract		Vitreous extract	
Substrate	µg.	µg.	µg.	µg.
Water	0	2	15	27
1 p.c. glycogen	3	27	118	132
0·01 M glucose	2	8	15	34
0·01 M glucose-1-phosphate	5	19	113	132
0·01 M glucose-6-phosphate	19	62	174	195
0·01 M fructose-1:6-diphosphate	46	304	120	283
	Nacreous homogenate		Vitreous homogenate	
	µg.	µg.	µg.	µg.
Water	1	10	9	14
1 p.c. glycogen	2	13	8	25
0·01 M glucose	0	10	7	34
0·01 M glucose-1-phosphate	7	18	20	12
0·01 M glucose-6-phosphate	22	43	30	17
0·01 M fructose-1:6-diphosphate	71	223	177	180

The slight increase with glucose indicates that there is a hexokinase reaction occurring but this could not be confirmed in acetone dried muscle by estimating the labile P transferred from ATP to glucose-6-phosphate (Ochoa, 1941). Experiments with all the substrates were also carried out at 25° C. to eliminate the possibility that the effects observed were caused by working at a temperature too far removed from what can be regarded as normal for this animal; essentially similar results were found but on a much reduced scale.

Phosphate Esterification.

The aqueous extracts of both parts of the muscle contain ATP-ases (Humphrey, 1949 a) which produce adenylic acid and adenosinediphosphate from ATP. Therefore the amount of ATP present at any time is a balance between the loss due to phosphorylation and ATP-ase action and the gain from glycolytic resynthesis; under experimental conditions there is usually a slight overall loss of ATP P_7. The higher content of ATP-ase in the nacreous tissue as compared to the vitreous part means that preparations from the nacreous portion would have a lower level of ATP on this account; this could be offered as a reason why nacreous tissue exhibited less active glycolysis than the vitreous tissue. However the fact that under conditions where glycolysis occurs equally well in both parts (i.e. fructose-diphosphate is the substrate) there is the same gain in ATP (as judged by P_7 values after correction for hydrolysis of fructose-diphosphate) for the two types of tissue, means that it is a deficiency in the glycolytic process and not more active ATP-ases which causes the nacreous tissue to exhibit less acid-production than the vitreous tissue when substrates such as glycogen are used (Table 4).

Uptake of phosphate into organic combination is seen best with homogenates; the gain in ester P ($P_{total} - P_7$) and loss of P_7 is increased by incubating in air or O_2 as compared with N_2 (Table 5).

TABLE 4.

Phosphate changes during glycolysis.

Extracts incubated 130 minutes under N_2 in the presence of 0·005 M $MgCl_2$, 0·001 M DPN, 0·002 M ATP and 0·001 M phosphate; 0·3 ml. extract in a final volume of 1·8 ml. The figures give the changes in ATP P_7.

	1 p.c. glycogen	0·02 M fructosediphosphate
Nacreous part.	—162 μg.	76 μg.
Vitreous part.	— 18	75

TABLE 5.

Effect of oxygen on phosphorylation.

Homogenates incubated 120 minutes in the presence of 0·005 M $MgCl_2$, 0·0015 M DPN, 0·003 M ATP, 1 p.c. glycogen and 0·001 M phosphate; 200 mg. muscle in a final volume of 1·5 ml.

	N_2	air	O_2
Nacreous part.	μg.	μg.	μg.
Change in P_7	—127	—150	—165
Change in P_{ester}	—13	0	+45
Vitreous part.			
Change in P_7	—85	—90	—93
Change in P_{ester}	—22	0	+56

Fractionation of Phosphorylated Intermediaries.

To determine which phosphorylated breakdown products of glycogen were normal constituents of the muscle, Ba salts were prepared and fractionated according to the methods of Le Page and Umbreit (1945). The results are set out in Table 6.

TABLE 6.

Presence of phosphorylated compounds.

The concentration of the substances is given as μM.

	Nacreous tissue	Vitreous tissue
Inorganic phosphate	7,890	9,260
Organic phosphate	9,764	12,440
ATP + ADP	422	732
Glucose-1-phosphate	20	47
Glucose-6-phosphate	5,120	6,010
Fructose-6-phosphate	182	173
Fructose-1:6-diphosphate	134	187
Triose phosphate	206	1,010
Phosphoglyceric acid	2,130	1,940
Phosphopyruvic acid	1,020	960

Thus both parts of the muscle contain similar concentrations of glycolytic intermediates, despite the difference in glycolytic intensity. The explanation for the disparity in glycolytic rates must therefore be sought in the enzymes which connect these compounds, particularly those acting between glycogen and fructose-diphosphate.

Synthesis of Glycogen.

The vitreous tissue contains more glycogen than the nacreous part of the muscle, representative figures being 0·74 p.c. as compared with 0·55 p.c. Also the vitreous tissue has a slightly more powerful glycogenase (Table 7).

TABLE 7.

Glycogenase in the muscle.

250 mg. muscle brei incubated 150 minutes in the presence of 0·01 M phosphate (pH = 7·3) and 1·7 p.c. glycogen: final volume = 2 ml.

	Glycogen used
Nacreous part	4·86 mg.
Vitreous part	6·40 mg.

Incubating muscle brei with glucose-1-phosphate led to synthesis of glycogen; fluoride was added to depress the breakdown of glycogen but this was only partly successful (Table 8). The nacreous tissue produced more glycogen than the vitreous tissue.

TABLE 8.

Production of glycogen.

500 mg. muscle brei incubated 150 minutes in the presence of 0·002 M phosphate (pH = 7·3), 0·03 M NaF and 0·03 M glucose-1-phosphate; final volume = 2·4 ml. The figures are the mg. glycogen in each tube.

	Nacreous tissue	Vitreous tissue
Initial	1·80	2·41
150 minutes (no substrate)	1·62	2·12
150 minutes (substrate)	5·16	2·68

Formation of Hexosephosphates.

Production of glucose-6-phosphate from glucose-1-phosphate was determined by estimating the change in P_T in muscle extracts on incubation (Table 9). These results show that both parts of the muscle have the same ability to carry out this change.

With glucose-6-phosphate as the substrate, fructose-6-phosphate and fructose-1:6-diphosphate were formed if iodoacetic acid were present to prevent further breakdown. This was shown by bringing the ice-cold trichloracetic acid filtrate to pH 8·2 and fractionating the Ba salts as previously carried out. Fructose was then determined in the fractions containing the esters (Table 10). The results show that here, the nacreous tissue is much less active than the vitreous.

TABLE 9.

Synthesis of Hexosemonophosphate.

0·6 ml. extract were incubated 60 minutes in the presence of 0·01 M $MgCl_2$ and 0·005 M glucose-1-phosphate; final volume = 1·2 ml.

	Nacreous part	Vitreous part
	µg.	µg.
Initial P_7	180	189
Final P_7	43	47
Change in P_7	—137	—142

TABLE 10.

Synthesis of fructosephosphates.

1 ml. extract incubated 120 minutes with 0·015 M glucose-6-phosphate, 0·01 M iodoacetic acid, 0·002 M ATP and NaOH to bring the pH to 7·5. The figures are the amount of ester formed per 2 ml. reaction mixture (≡ 1 ml. extract).

	Nacreous part	Vitreous part
	µg.	µg.
Fructose-6-phosphate	69	580
Fructose-1:6-diphosphate	270	442

Presence of Phosphagen.

Studies on other species point to the presence of arginine phosphate in oyster muscle (Meyerhof, 1928) and it proved possible to demonstrate this compound in both the nacreous and vitreous tissue (Table 11) by methods involving phosphorus determination after fractionating the Ca salts (J. Needham, D. M. Needham, Baldwin and Yudkin, 1932). These results were confirmed in other experiments by hydrolysing the Ca salt of arginine phosphate with 0·1 M HCl at 100° C. and determining the arginine set free by using arginase to produce urea, which could be estimated manometrically with urease (Krebs, 1942).

TABLE 11.

Estimation of arginine phosphate.

Muscle ground under ice-cold conditions with sand and 10 ml. 10 p.c. trichloracetic acid/gm. The values given relate to 10 gm. muscle.

	Nacreous tissue	Vitreous tissue
Ca salt fraction	1·4 mg. P	1·6 mg. P
Extract after hydrolysis at 28° C.	2·5 mg. P	3·0 mg. P
Arginine phosphate	1·1 mg. P	1·4 mg. P
mg. p.c. arginine phosphate	90	114

The content of phosphagen did not fall significantly when oysters were left out of the water for a few days. However, as noted before (Humphrey, 1949 a) the amount of ATP decreased and the amount of adenylic acid increased. To compare individual oysters in a series, it is necessary to calculate the ratio between these two constituents as the absolute amounts vary from one animal to another. The results in Table 12 show that the ratio of ATP to adenylic acid fell as the organism was called on to do more work by maintaining shell-closure against the pull of the elastic ligament.

TABLE 12.

Change in ATP concentration.

The oysters were left out of water at room temperature (about 20° C.); the values given are ATP/adenylic acid, both expressed as mg. p.c.

Days	Nacreous tissue	Vitreous tissue
0	2·0	2·4
7	0·5	0·8

Acid Production by Whole Muscle.

There is no pyruvic acid in muscle tissue taken directly from the animal; the content of lactic acid under those conditions was variable and ranged from 2·6 to 18·0 mg. p.c. for the nacreous part (mean = 8·4) and 2·4 to 18·0 mg. p.c. for the vitreous part (mean = 7·3). In most cases the nacreous tissue contained more than the vitreous tissue.

Determination of the buffer capacity showed that in the region where the pH was directly proportional to the amount of base added, the buffer capacity of the nacreous tissue was 120 micro-equivalents/gm./pH unit whereas the corresponding value for the vitreous tissue was 234 micro-equivalents (Table 13).

TABLE 13.

Buffer capacity.

1 gm. muscle ground with sand and 9 ml. H_2O; 0·25 N HCl was added and the pH determined after 5 minutes by the glass electrode. The figures given represent the pH values when 800 mg. nacreous and 742 mg. vitreous tissue were used.

	Nacreous tissue	Vitreous tissue
0 ml. acid	6·52	6·81
0·1 ml. acid	5·31	5·87
0·2 ml. acid	4·40	5·42
0·3 ml. acid	4·01	5·10
0·4 ml. acid	3·65	4·87
0·5 ml. acid	3·43	4·78
0·7 ml. acid	2·89	4·27

This would indicate that the vitreous part is better adapted in this regard to withstand acid accumulation. Attempts to increase the acid content of the muscle by fatiguing with weights suspended from the shell or by electrical stimulation, met with only limited success.

Weights of up to 15 lb. were hung onto the shell for periods up to 12 hours and the animal kept either in the air or in aerated sea-water; in a few cases the muscle was ruptured

due to the valves being torn apart. In the experiments involving electrical stimulation both interrupted direct current (6 volts, 50 pulses per second) and current from an inductorium (primary voltage 1·5 volts, secondary voltage 2 to 40 volts at 40–50 cycles) were used; nichrome electrodes were applied directly to the muscle. In both of these types of experiments there was no pyruvic acid produced and the content of lactic acid was within the range obtained on control muscles.

DISCUSSION.

Glycolytic Pattern.

When compared to the glycolytic ability of rabbit muscle, oyster adductor muscle is several hundred times less powerful. Ritchie (1928) gives a list of the amounts of lactic acid produced by a number of different species in conditions such as fatigue, rigor and also when minced muscle is incubated in alkaline phosphate. Allowing for the variety of different investigators and the experimental methods used, the oyster is again deficient in this ability even when compared to other invertebrate species. Working under similar conditions (Humphrey, 1949b; Humphrey and Siggins, 1949) Q values (total acid on mg. dry wt. basis) of 0·67 for the cockroach and 2·0 for the grasshopper are much higher than the figures of 0·06 for nacreous tissue and 0·20 for vitreous tissue. The question of the occurrence of pyruvic acid as an end-product in these species has been discussed previously (Humphrey, 1949b).

Neither part of the adductor muscle shows true cross-striations in the same sense as frog or rabbit muscle does. Therefore it is best to regard the different parts of the adductor as specialized tissue not comparable either with smooth or skeletal muscle of other animals. Plenk (1924) reviews the opposing views on the question as to whether various lamellibranch muscles show any striations at all.

Biochemically, the most marked difference between the two parts of the adductor muscle is the failure of the nacreous tissue readily to bring about the conversion of glycogen to fructose-diphosphate; this is responsible for the weaker glycolytic ability of this part when compared to the vitreous tissue. An analysis of the steps intermediate between these two compounds (Tables 9 and 10), shows that it is the breakdown of glucose-6-phosphate which is deficient, the other steps being carried out equally well by both tissues. Further, the equilibrium between glucose-1-phosphate (in the presence of fluoride) and glycogen gives more glycogen synthesis with nacreous than with vitreous tissue. The results in Table 5 show that oxidative phosphorylation can occur in both parts of the muscle.

The fractionation of the Ba salts of the phosphate esters shows that the substrates which can be used for glycolysis are normal constituents of the muscle. When compared with similar investigations on rat (Le Page, 1946) and dolphin (Du Bois, Geiling, McBride and Thomson, 1948) tissues, it is

seen that oyster muscle contains an unusually high proportion of glucose-6-phosphate. For many other invertebrate tissues Baldwin and Needham (1937) and Baldwin (1938) have shown how these phosphorylated compounds can interact in a fashion similar to that found for mammalian tissues.

Energy Source of the Muscle.

Parnas (1910) has shown that during working of lamellibranch adductor muscles by loading with weights, there is no increase in O_2 consumption or CO_2 output. Nozawa (1929) states that in loading there is a 20–30 p.c. increase in O_2 consumption but his figures do not support this; if anything, they show an initial increase followed by a decrease, the net effect being no change. With experiments involving the fixing of weights on to the shell, there is always the possibility that resulting effects are in part due to the handling of the animal during the attaching of wires and hooks, or boring holes in the shell. Since the muscle (certainly the nacreous part and possibly also the vitreous part) is functioning all the time an oyster is out of water, by resisting the pull of the elastic ligament, the fact that the content of ATP falls when the animal is left out of water means that ATP is being broken down to provide the energy for the muscle. Here, there is no question of interference with the animal. These conditions are abnormal in that there is no supply of sea-water running continuously through the shell; also adequate resynthesis of ATP does not occur through significant phosphagen breakdown or glycolysis. If ATP is then to be regarded as the primary energy source of the muscle and glycolysis the main mechanism by which it is formed, in view of the low glycolytic rates of both parts of the muscle, the process of glycolysis must go on continuously in order to keep up the supplies of ATP for intermittent activity. This would distinguish this type of muscle sharply from mammalian skeletal muscle where glycolysis is a temporary process brought into play after contraction.

The supposition that glycolysis in oyster muscle is a slow continuous process is supported by the fact that under aerobic conditions glycolysis proceeds at an undiminished rate; in addition, oxidative processes occur leading in part to oxidative phosphorylation (Table 5).

SUMMARY.

Homogenates and extracts of the nacreous and vitreous tissue of the adductor muscle of *Saxostrea commercialis* produce pyruvic and lactic acids.

In the homogenate the nacreous tissue produces 0·88 μl. acid/mg.N/hour and the vitreous tissue 4·4 μl.

Glycogen, glucose-1-phosphate, glucose-6-phosphate, fructose-diphosphate and to some extent, glucose, can all serve as substrates for acid production.

In O_2, the muscle can build up organic phosphate from ATP and glycogen.

The presence in the muscle of several phosphorylated breakdown compounds of glycogen was shown by fractionation of the Ba salts.

Both parts of the muscle could synthesize glycogen from glucose-1-phosphate, the nacreous tissue being more powerful in this regard.

Glucose-1-phosphate is converted into glucose-6-phosphate with equal facility by both parts of the muscle but further breakdown to fructose phosphates is much more readily effected by the vitreous, than the nacreous tissue.

When oysters are left out of water there is a decrease in the amount of ATP in the muscle and it is concluded that this breakdown provides the energy for the muscle to resist the pull of the ligament. The hypothesis is advanced that regeneration of ATP proceeds through glycolysis which goes on continuously under both aerobic and anaerobic conditions.

REFERENCES.

Baldwin, E. (1938): Biochem. J., 32, p. 888.
Baldwin, E. and Needham, D. M. (1937): Proc. Roy. Soc. Lond. Ser. B, 122, p. 197.
Du Bois, K. P., Geiling, E. M. K., McBride, A. F. and Thomson, J. F. (1948): J. gen. Physiol., 31, p. 347.
Friedemann, T. E. (1938): J. biol. Chem., 123, p. 161.
Humphrey, G. F. (1941): Austral. J. exp. Biol., 19, p. 311.
Humphrey, G. F. (1944): *Ibid.*, 22, p. 135.
Humphrey, G. F. (1946): *Ibid.*, 24, p. 261.
Humphrey, G. F. (1949 a): Physiol. comp. oecol., 1, p. 366
Humphrey, G. F. (1949 b): J. cell. comp. Physiol., 34, p. 323.
Humphrey, G. F. (1950): in press.
Humphrey, G. F. and Siggins, L. (1949): Austral. J. exp. Biol., 27, p. 353.
Krebs H. A. (1942): Biochem. J., 36, p. 758.
Krebs, H. A. and Johnson, W. A. (1937): *Ibid.*, 31, p. 645.
Le Page, G. A. (1946): Amer. J. Physiol., 146, p. 267.
Le Page, G. A. and Umbreit, W. W. (1945): Manometric Techniques and related Methods for the Study of Tissue Metabolism. Burgess Publishing Co., Minneapolis.
Marceaus, F. (1909): Arch. Zool. exp. gén. Sér. 5, 2, p. 295.
Meyerhof, O. (1928) Arch. Sci. biol., 12, p. 536.
Needham, J., Needham, D. M., Baldwin, E. and Yudkin, J. (1932): Proc. Roy. Soc. Lond. Ser. B, 110, p. 260.
Nozawa, A. (1929): Sci. Rep. Tohoku Imp. Univ. Ser. 4, 4, p. 315.
Ochoa, S. (1941): J. biol. Chem., 41, p. 245.
Parnas, J. K. (1910): Arch. ges. Physiol., 134, p. 441.
Plenk, H. (1924): Z. wiss. Zool., 122, p. 1.
Ritchie, A. D. (1928): The comparative Physiology of muscular Tissue. Cambridge University Press.

THE LYMPHATIC DRAINAGE OF PLASMA FROM THE PERITONEAL CAVITY OF THE CAT

by F. C. COURTICE AND A. W. STEINBECK[1]

(From the Kanematsu Memorial Institute of Pathology, Sydney Hospital, Sydney).

(Accepted for publication 11th October, 1949.)

Investigations of peritoneal drainage have been mainly confined to the pathways of absorption of inert particles, bacteria or erythrocytes (MacCallum, 1903; Buxton and Torrey, 1906; Bolton, 1921; Cunningham, 1922; Florey, 1927; Higgins and Graham, 1929; Allen, 1935; Simer, 1944 and 1948). These authors describe how rapidly particles are absorbed by the diaphragmatic lymphatics while absorption by other parts of the peritoneum is much slower. Much controversy has arisen concerning the question of stomata in the diaphragmatic peritoneum whereby particles of varying size may gain entry to the lymphatic capillaries just deep to the peritoneum. Once the particles enter the diaphragmatic lymph capillaries all are agreed that the main pathway is then in the anterior mediastinum in close proximity to the mammary vessels. Higgins and Graham describe five routes, but maintain that 80 p.c. of the material absorbed passes by this route. The lymph channels then pass through the anterior mediastinal lymph nodes from which some authors assert that the lymph then goes to the thoracic or right lymph ducts.

In experimental studies on the absorption of fluids from the peritoneal cavity, however, Watkins and Fulton (1938) and Florey and Witts (1928) have cannulated the thoracic duct only, and have based their conclusions on the apparent assumption that all or nearly all the lymph from the peritoneal cavity passes into the thoracic duct. Since we proposed to investigate the rate of absorption of fluids from the peritoneal cavity, it was decided that the final lymphatic pathways should first be studied in more detail.

METHODS.

Cats, anaesthetized with nembutal, were used in these experiments. The fluid injected into the peritoneal cavity was 2 ml. cat's heparinized plasma/kg. body weight to which had been added 4 mg./kg. of the dye, T1824 (Evans Blue). In these proportions the dye is all combined with plasma protein, mainly albumin (Rawson, 1943; Courtice and Simmonds, 1949). Provided the dye remains combined with the albumin, the determination of dye absorption

[1] This work was carried out under a grant from the National Health and Medical Research Council, Canberra.

will give a measure of the albumin absorption. To ensure that the dyed-plasma was all introduced into the peritoneal cavity and not into the gut, a small incision was made in the skin of the abdominal wall. The muscle and peritoneum were then held up away from the gut with fine forceps and the plasma injected through a blunt needle.

In some experiments, no lymph ducts were cannulated, the rate of absorption of dye-protein being determined merely by estimating the dye concentration in the plasma after intraperitoneal injection. In others, the thoracic duct was cannulated and the right lymph duct was left intact, ligated or cannulated. The right lymph duct was cannulated just above the apex of the pleura without opening the thorax. When these lymph ducts were cannulated, the lymph was collected continuously for a period of five hours in most cases. Dry heparin was introduced into the lymphatic cannula at intervals to prevent coagulation.

One femoral artery was cannulated to obtain blood samples for the estimation of the dye concentration in the plasma. In some cases the trachea was also cannulated, so that, with the aid of valves, the animal could breathe 5 p.c. CO_2 in oxygen or room air as desired.

The dye concentration in 1 ml. samples of the plasma and lymph was always first extracted with n-butyl alcohol (Harington, Pochin and Squire, 1940) before being estimated with a Spekker photoelectric absorptiometer (Adam Hilger Ltd.). In this absorptiometer a filter combination, Ilford 607 and a Calorex heat filter, was used. Without extraction it is impossible to estimate the dye concentration, especially in the thoracic duct lymph where the opalescence varies considerably over a period of 5 hours. The milkiness of the plasma may also vary greatly. Before the thoracic duct is cannulated, the plasma may be quite milky, whereas an hour after cannulation it is usually clear, the fat having been deposited.

To show whether the dye is absorbed attached to the injected protein, rabbits were used. Human dyed plasma was injected intraperitoneally, and anti-human rabbit serum employed to determine the absorption of human albumin by means of a precipitin reaction. The precipitin method used was that described by Courtice and Simmonds (1949).

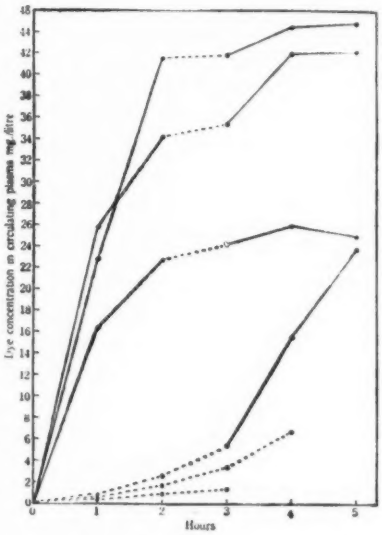

Fig. 1. The concentration of dye in the plasma after the intraperitoneal injection of 2 ml./kg. heparinized cat's plasma + 4 mg./kg. T-1824 in the anaesthetized cat.
----- Room air breathed.
—— 5 p.c. CO_2 in oxygen breathed.

RESULTS.

Experiments with no Lymph Ducts Cannulated.

In the first group of experiments no lymph ducts were cannulated. The dye concentration in the plasma was taken as a measure of the rapidity of absorption of the albumin-dye complex. The anaesthetized cats were in the horizontal supine position and after intraperitoneal injection of the dyed plasma, they breathed either room air or 5 p.c. CO_2 in oxygen.

The CO_2 increased both the rate and depth of respiration considerably. In some cases room air was breathed throughout while in others room air was breathed for 3 hours followed by 5 p.c. CO_2 for 2 hours, or 5 p.c. CO_2 was breathed for 2 hours followed by room air for 1 hour and then 5 p.c. CO_2 again for a further 2 hours.

The results of these experiments are shown in Fig. 1. The effect of increased respiratory rate and depth on the absorption is evident. The increased rate of absorption when 5 p.c. CO_2 is breathed is probably due partly to the increased rate and degree of contraction of the diaphragm and also to the increased pressure changes in the peritoneal cavity.

That the dye level in the plasma gives a measure of the albumin absorption has been determined by experiments in rabbits in which dyed human plasma was injected intraperitoneally. The dye concentration in the plasma was determined at intervals together with the concentration of human plasma proteins by a precipitin reaction. The results of these experiments are shown in Table 1. The dye concentration in the plasma is represented by the extent of dilution of the original dye concentration in the plasma introduced into the peritoneal cavity. The human plasma protein is also represented by the degree of dilution of this protein in the rabbit plasma. For example, the dye concentration in the fluid injected was 2,000 mg. per litre. If the dye concentration in the rabbit plasma is 10 mg./litre, the dye has been diluted 1/200. For the dilution of the protein, the original plasma was made up in many dilutions with saline and a positive precipitin reaction just observed at a dilution of 1/8,000. If the titre in the rabbit plasma is now found to be 1/40, the original human plasma must have been diluted 1/200.

TABLE 1.

The concordance of the plasma concentrations of T-1824 and heterologous (human) labelled plasma after an intraperitoneal injection of dyed human plasma, in the rabbit. The results are expressed as dilutions of the original dye and protein concentrations of the injected fluid.

Sampling period	Dilution of original					
	Exp. 1		Exp. 2		Exp. 3	
Hour	T-1824	Human Plasma	T-1824	Human Plasma	T-1824	Human Plasma
0	—	—	—	—	—	—
1	1/2,500	1/2,000	1/270	1/200	1/556	1/800
3	1/54	1/80	1/49	1/48	1/84	1/120
5	1/45	1/48	1/26	1/30	1/95	1/120
8	1/46	1/48	1/30	1/27	1/75	1/120
24	1/91	1/120	1/53	1/34	1/56	1/30
48	1/167	1/120	—	—	—	—

The figures in Table 1 show that the dilutions of the dye and of the human protein agree very closely, especially when it is considered that the precipitin test is not as accurate quantitatively as the estimation of the dye. It appears, therefore, that the dye and the protein are absorbed together and not split beforehand. The estimation of the dye concentration in blood and lymph should, therefore, give an accurate measure of the absorption of the albumin.

Absorption by the Thoracic Duct.

In this series of experiments the thoracic duct was cannulated before the intraperitoneal injection of the dyed plasma, and the lymph collected for 5 hours after injection. Blood samples were also taken hourly to give an indication of the amount of albumin-dye complex absorbed other than by this duct. In some experiments the animal breathed room-air throughout, in others 5 p.c. CO_2 in O_2 for 2 hours, room air for 1 hour and 5 p.c. CO_2 for a further 2 hours. The volume and dye concentration of the lymph were determined and from these figures the total dye absorbed was calculated. The dye concentration was also estimated in the plasma, and assuming a plasma volume of 35 ml./kg. in the cat (Courtice and Gunton, 1949) the amount of dye in the plasma at any time could be determined. This latter figure must always be less than the amount absorbed into the blood, because some will leave the circulation and pass into the tissues. Some of this will also pass back into the thoracic duct, but considering the high dye concentration in the lymph compared with that in the plasma, this entry of dye into the thoracic duct other than from the peritoneal cavity will not be large (Courtice, 1943). So the figures for thoracic duct absorption will be somewhat too high while those in the plasma will be too low. These experiments will give, however, an approximate indication of the amount of albumin-dye absorbed from the peritoneal cavity by the thoracic duct and by other routes. In Fig. 2 the dye absorbed is expressed as a percentage of that injected, viz. 4 mg./kg.

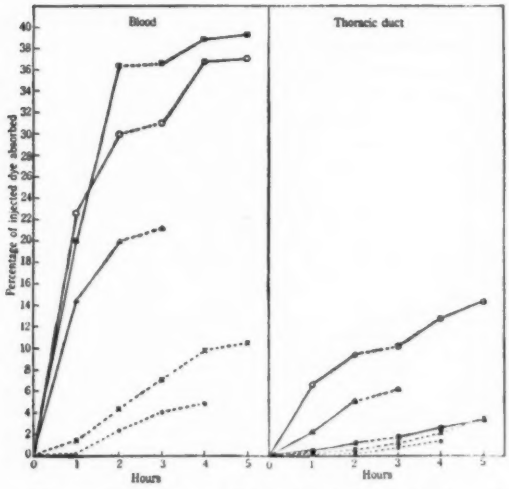

Fig. 2. The amount of dye absorbed by the thoracic duct and by other routes into the blood after the intraperitoneal injection of dyed-plasma as in Fig. 1. Results expressed as a percentage of the dye injected.

----- Room air breathed.
—— 5 p.c. CO_2 in oxygen breathed.

Two facts are evident from these results. Firstly, the amount absorbed by other routes is very much greater than by the thoracic duct, and secondly, the increased respiratory movement produced by CO_2 increases the absorption both by the thoracic duct and by the other routes.

Absorption by the Thoracic and Right Lymph Ducts.

At *post mortem* in these earlier experiments, it could be seen that dye was passing from the anterior mediastinal lymph nodes to the right side into the right lymph duct. It was, therefore, decided to cannulate both thoracic and right lymph ducts before the intraperitoneal injection of dyed plasma. The right lymph duct of the cat, as of the dog, varies considerably in its anatomical arrangement. In some cases there are many small ducts which cannot be

cannulated, but in others there is one main duct which can be cannulated while smaller vessels, if present, can be ligated. It is, therefore, not possible to cannulate the right lymph duct in every case as it is with the thoracic duct, and the size of the cat bears no relation to the ease with which the right lymph duct can be cannulated. For this reason, different experiments were planned to give essentially the same information. In some cases the right lymph duct was cannulated before the injection of the dyed plasma, in others it was cannulated after the injection when it could more easily be seen by its blue colour, while in those animals in which the right lymph duct consisted of several small ducts, these were tied off and so lymph was prevented from entering the blood on the right side.

As with dogs, the connection between the thoracic and right lymph ducts varies considerably. The thoracic duct lymph was always milky, but the right lymph duct lymph varied, sometimes being slightly milky and sometimes quite clear. In one animal the right lymph duct lymph was clear while room air was breathed, but became somewhat milky when 5 p.c. CO_2 was breathed and respiration became more forceful.

TABLE 2.

Typical experiments in which cat breathed 5 p.c. CO_2 in O_2 for first two hours, room air for third hour and 5 p.c. CO_2 in O_2 for last two hours, 4 mg. dye/kg. in 2 ml. plasma/kg. injected intraperitoneally at zero hour in each case.

Time Hour	Thoracic duct lymph			R.L.D. lymph			Plasma dye conc. mg./l.
	Volume ml.	Dye conc. mg./l.	Total dye mg.	Volume ml.	Dye conc. mg./l.	Total dye mg.	
Cat 6 2·95 kg.							
0 – ½	4·4	100·0	0·440	R.L.D. intact throughout			—
½–1	3·3	103·0	0·340				25·8
1 –1½	3·0	77·7	0·233				—
1½–2	1·5	72·7	0·109				34·2
2 –3	1·9	42·5	0·081				35·4
3 –3½	3·4	48·7	0·166				—
3½–4	1·9	75·0	0·143				42·0
4 –4½	1·6	68·5	0·110				—
4½–5	1·2	74·5	0·089				42·2
Cat 10 2·38 kg.							
0 – ½	3·4	8·8	0·027	R.L.D. tied off throughout			—
½–1	2·7	58·5	0·158				0·3
1 –1½	2·5	69·0	0·172				—
1½–2	2·2	68·8	0·151				0·7
2 –3	2·8	46·3	0·130				1·3
3 –3½	2·4	58·5	0·140				—
3½–4	1·9	87·0	0·165				1·5
4 –4½	1·7	86·0	0·146				—
4½–5	1·4	86·0	0·120				1·8
Cat 11 3·86 kg.							
0 – ½	3·6	9·0	0·032	2·2	148	0·326	—
½–1	3·3	92·3	0·305	1·7	450	0·765	0·05
1 –1½	3·3	155·0	0·512	1·8	522	0·939	—
1½–2	2·8	183·5	0·514	1·1	513	0·565	0·7
2 –3	2·8	127·5	0·357	0·6	369	0·221	1·3
3 –3½	3·1	81·2	0·252	1·3	363	0·472	2·0
3½–4	2·5	88·5	0·221				
4 –4½	1·5	77·5	0·116	1·1	322	0·354	2·5
4½–5	2·1	74·3	0·156				

The results of three typical experiments are given in Table 2. In these experiments, the thoracic duct was cannulated and 5 p.c. CO_2 was breathed for 2 hours, air for 1 hour and 5 p.c. CO_2 for 2 hours in all three. In the first experiment the right lymph duct was left intact, in the second it was tied off and in the third cannulated. The absorption by the thoracic duct was essentially the same in all three cases, the rate of absorption falling during the third hour when room air was breathed. The dye concentration in the plasma rose considerably when the right lymph duct was intact, but there was only a very small rise when the right lymph duct was ligated or cannulated. In the third experiment it is also evident that the absorption of dye by the right lymph duct is greater than by the thoracic duct and that respiration affects the absorption from the right lymph duct.

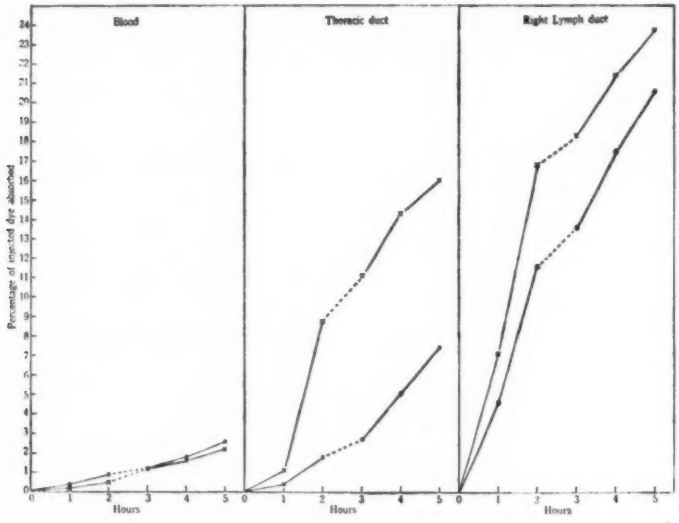

Fig. 3. The amounts of dye absorbed by the thoracic and right lymph ducts and by other routes after the intraperitoneal injection of dyed-plasma as in Fig. 1. Results expressed as a percentage of the dye injected.

----- Room air breathed.
—— 5 p.c. CO_2 in oxygen breathed.

In two experiments in which both thoracic and right lymph ducts were cannulated before the intraperitoneal injection of dyed plasma, the amount of dye absorbed by the blood, thoracic duct and right lymph duct has been calculated. This is represented in Fig. 3 as a percentage of the dye injected. In these experiments some dye does appear in the blood stream, but it is only a small proportion of that collected from the thoracic and right lymph ducts. As with experiments on the lymph drainage of the pleural cavity (Courtice and Simmonds, 1949) it is difficult to ascertain how this dye enters the blood stream. It is possible that it may do so by small lymph vessels which have not been cannulated or ligated.

These experiments show that nearly all the albumin-dye complex can be collected from the thoracic and right lymph ducts and it is probable that all

is absorbed by these lymphatic channels. In all experiments in which the thoracic duct has been cannulated and the right lymph duct either left intact or cannulated, the relative proportions of dye absorbed by the thoracic duct and right lymph duct can be calculated. In those experiments in which the right lymph duct has not been cannulated, the dye level in the plasma and an assumed plasma volume will give an approximate figure for the absorption by the right lymph duct. In Table 3 these figures show that over a period of 5 hours under varying conditions of respiration the amount of dye absorbed by the thoracic duct is always considerably less than that absorbed by the right lymph duct, the proportions being very similar to those obtained for the pleural cavity by Courtice and Simmonds (1949). The actual ratio in the last column is probably somewhat higher than that given, because the amount of dye in the plasma is lower than that actually absorbed since in 5 hours some will pass into the tissues, and when the right lymph duct is cannulated some small vessels may be ligated, thus reducing the amount of lymph which would normally flow by this route.

TABLE 3.

The amount of dye absorbed in 5 hours by the thoracic duct and by other routes after the intraperitoneal injection of 4 mg. dye/kg. in 2 ml. plasma/kg. in the cat. The figures are expressed as a percentage of the amount of dye injected. The thoracic duct was cannulated for 5 hours in each case. In the first 5 experiments the cat breathed 5 p.c. CO_2 for 2 hours, air for 1 hour and 5 p.c. CO_2 for 2 hours, while in the last two experiments, air was breathed throughout.

Cat No.	Remarks	Thoracic duct	Blood plasma	R.L.D.	R.L.D. + blood	R.L.D. + blood / thoracic duct
8	R.L.D. cannulated for 5 hours	7·4	2·6	20·6	23·2	3·1
11	R.L.D. cannulated for 5 hours	16·1	2·2	23·7	25·9	1·6
3	R.L.D. cannulated from 3–5 hours only	10·9	21·8	8·5	30·3	2·8
6	R.L.D. intact	14·5	37·0	—	37·0	2·6
5	R.L.D. intact	3·4	39·3	—	39·3	11·5
7	R.L.D. intact	3·6	10·5	—	10·5	2·9
1	R.L.D. intact	1·4	4·8	—	4·8	3·4

Although this arrangement is the general rule, there may be exceptions. We have had in this series two such cases. In one case, the thoracic duct was cannulated and the cat allowed to breathe 5 p.c. CO_2 for 2 hours, air for 1 hour and 5 p.c. CO_2 for 2 hours. In this time 59·9 p.c. of the injected dye was absorbed by the thoracic duct, while only 8·6 p.c. was accounted for by the rise in dye concentration in the plasma. At *post mortem* it was seen that the vessels from the anterior mediastinal nodes which usually pass to the right side passed to the left and joined the thoracic duct. Only a few small vessels passed to the right. In another case in which we failed to cannulate the right lymph duct, at *post mortem* a mass of very blue ducts entered the vein on the left side close to the entrance of the thoracic duct, but the entries of these ducts were definitely separate. On the right side only one very small blue

lymph vessel could be seen. The experimentalist has, therefore, to be prepared for a variety of arrangements of what is called the right lymph duct. The thoracic duct, on the other hand, although it may vary in the detail of its entrance into the vein, can always be cannulated.

DISCUSSION.

It is evident from experiments on the anaesthetized cat that by far the greater part of plasma protein introduced into the peritoneal cavity is absorbed by the diaphragmatic lymphatics and then passes into the blood stream mainly through the right lymph duct. The remainder is absorbed by the lymphatics of the mesentery, omentum and the parietal and visceral peritoneum, and most of this passes into the thoracic duct. The right lymph duct lymph as well as carrying more of the dye-protein than the thoracic duct lymph also becomes blue more quickly. The ratio of dye absorbed by the right lymph duct divided by dye absorbed by the thoracic duct is much greater in the first two half-hour periods than it is later. This indicates that the dye-protein rapidly passes into the diaphragmatic lymphatics and then via the parasternal lymphatics mainly to the right lymph duct, while the passage through the omentum, mesentery and parietal peritoneum and then into the thoracic duct is slower.

The effect of respiratory movement on absorption is considerable. The absorption from the peritoneal cavity of the anaesthetized cat breathing air is probably due partly to the effect of anaesthesia on respiration rate and also partly due to the recumbent supine position. In the rabbit anaesthetized initially and then allowed to recover and attain the upright position, the increase in dye concentration in the plasma is comparable with that in the anaesthetized cat breathing 5 p.c. CO_2, while continuous anaesthesia in the rabbit slows down absorption somewhat, but not to the same extent as in the cat. It seems, therefore, that the rate of absorption in an anaesthetized cat breathing 5 p.c. CO_2 gives a better indication of absorption from the normal animal than does that from an anaesthetized cat breathing room air. It was for this reason that in many of these experiments the respiration was increased by the inhalation of 5 p.c. CO_2.

The right lymph duct has been shown to play the major rôle in lymph drainage from the heart (Drinker and Yoffey, 1941), lungs (Drinker, 1945; Cameron and Courtice, 1946), pleural cavity (Courtice and Simmonds, 1949), and now it appears that most of the fluid drained by the lymphatics from the peritoneal cavity also passes by this route. This fact must be borne in mind when interpreting experimental results of the lymph drainage from any of these regions. In investigations of pulmonary oedema, for example, an increased lymph flow in the right lymph duct is no doubt due to an increased lymph flow from the oedematous area, but it must be remembered that this duct also drains these other regions and if free fluid exists in these cavities, it may be difficult to interpret the results.

SUMMARY.

The final lymphatic pathways of absorption of plasma introduced into the peritoneal cavity of the cat have been investigated.

Heparinized cat's plasma, 2 ml./kg., to which 4 mg./kg. of the dye T1824 was added, was injected into the peritoneal cavity and the absorption studied by determining the dye concentration in the plasma, thoracic duct and right lymph duct lymph.

The results show that the dye-albumin is practically all absorbed by the right lymph duct and thoracic duct, only a small percentage of the amount absorbed, about 5 p.c. entering the blood stream when these ducts are cannulated. This small amount may enter through small lymphatics which could not be ligated.

Of the amount entering by the lymphatics, the greater part passes through the diaphragmatic lymphatics to the right lymph duct via the parasternal lymph trunks. The ratio of the dye-albumin absorbed by the right lymph duct to that absorbed by the thoracic duct is on an average 4 to 1 over a 5-hour period. It is thus important to cannulate the right lymph duct as well as the thoracic duct, when investigating the lymphatic drainage from the peritoneal cavity.

Acknowledgment. We should like to thank Mr. J. Harding for his technical assistance in these experiments.

REFERENCES.

Allen, L. (1936): Anat. Rec., 67, p. 89.
Bolton, C. (1921): J. Path. Bact. 24, p. 429.
Buxton, G. H. and Torrey, J. C. (1906): J. med. Research, 15, p. 5.
Cameron, G. R. and Courtice, F. C. (1946): J. Physiol., 105, p. 175.
Courtice, F. C. (1943): *Ibid.*, 102, p. 290.
Courtice, F. C. and Gunton, R. W. (1949): *Ibid.*, 108, p. 405.
Courtice, F. C. and Simmonds, W. J. (1949): *Ibid.*, 109, p. 117.
Cunningham, R. S. (1922): Amer. J. Physiol., 62, p. 248.
Drinker, C. K. (1945): Pulmonary oedema and inflammation. Harvard University Press.
Drinker, C. K. and Yoffey, J. M. (1941): Lymphatics, lymph and lymphoid tissue. Harvard University Press.
Florey, H. W. (1927): Brit. J. exp. Path., 8, p. 479.
Florey, H. W. and Witts, L. J. (1928): Lancet, 1, p. 1323.
Harington, C. R., Pochin, E. E. and Squire, J. R. (1940): Clin. Sci., 4, p. 311.
Higgins, G. M. and Graham, A. S. (1929): Arch. Surg., 19, p. 453.
MacCallum, W. G. (1903): Bull. Johns Hopkins Hosp., 14, p. 105.
Rawson, R. A. (1943): Amer. J. Physiol., 138, p. 708.
Simer, P. H. (1944): Anat. Rec. 88, p. 175.
Simer, P. H. (1948): *Ibid.*, 101, p. 333.
Watkins, A. L. and Fulton, M. N. (1938): Amer. J. Physiol., 122, p. 281.

THE RATE OF ABSORPTION OF HEPARINIZED PLASMA AND OF 0·9 P.C. NACL FROM THE PERITONEAL CAVITY OF THE RABBIT AND GUINEA-PIG

by F. C. COURTICE AND A. W. STEINBECK[1]

(From the Kanematsu Memorial Institute of Pathology, Sydney Hospital, Sydney).

(Accepted for publication 11th October, 1949.)

The experiments described by Courtice and Steinbeck (1950) show that when plasma is injected intraperitoneally in the cat it is wholly or almost wholly absorbed by the lymphatics and that by far the greater proportion passes into the right lymph duct, the remainder entering the blood stream by the thoracic duct. In these experiments only a small volume of plasma was injected, 2 ml./kg., so the experiments give little indication of the absorption rate of large volumes of fluid. Bolton (1921) studied the absorption of ascitic fluid containing 7·5 p.c. solids and of 0·9 p.c. NaCl from the peritoneal cavity of the cat in short term experiments. He concluded that the ascitic fluid was evenly absorbed by the lymphatics whereas 0·9 p.c. NaCl was rapidly absorbed at first and then more slowly as the solid content increased. Watkins and Fulton (1938) measured the flow of thoracic duct lymph after the introduction of fairly large quantities of various fluids into the peritoneal cavity of dogs and found little if any increase in lymph flow. Since the thoracic duct drains only a minor part of the peritoneal fluid, and since the animals were in the recumbent, supine position and anaesthetized, it is probable that the results of these experiments are not a true indication of the absorption rate in the intact, unanaesthetized animal. Courtice and Simmonds (1949b) pointed out the effect of anaesthesia and the recumbent position in slowing absorption of dyed plasma from the pleural cavity and lungs. Respiration considerably affects absorption from the peritoneal cavity in the cat, and as absorption is mainly through the diaphragmatic lymphatics, it seems that the position of the animal may also affect the rate.

It was, therefore, proposed to investigate the absorption of a crystalloid solution (0·9 p.c. NaCl) and of a protein-rich fluid (heparinized plasma) from the peritoneal cavity of rabbits and guinea-pigs which were anaesthetized initially, but allowed to recover.

[1] This work was carried out under a grant from the National Health and Medical Research Council, Canberra.

METHODS.

Rabbits were anaesthetized initially with intravenous nembutal and guinea-pigs with ether. The rabbits came out of the anaesthetic in about an hour while the guinea-pigs recovered from the ether in a few minutes. The fluids were injected by making a small incision in the skin of the abdominal wall on the right side, holding the muscle and peritoneum up with a small pair of rat-tooth forceps and injecting the fluid through a blunt needle. In this way the large gut was avoided and the fluid could with certainty be introduced into the peritoneal cavity and not the intestine. The injection was made at the side to avoid leakage when the animal recovered and attained its normal posture.

In all cases 4 mg./kg. of the dye, T-1824, were injected and this was dissolved in heparinized plasma or 0·9 p.c. NaCl. Rabbit plasma was used for rabbits and guinea-pig plasma for guinea-pigs. The quantities of fluid injected were 6 and 20 ml./kg. in the rabbit and 20 ml./kg. in the guinea-pig.

An indication of the rate of absorption was obtained by determining the dye concentration in the circulating plasma at intervals after injection in the rabbit. In the guinea-pig this was impractical. To afford some overall indication of this rate, the dye concentration of the plasma in this animal was determined in a terminal cardiac plasma sample. The gross rate of absorption was determined by killing groups of both animals at stated times after the injection and collecting the peritoneal fluid with a pasteur pipette. The rabbits were killed with excess intravenous nembutal, the guinea-pigs with chloroform.

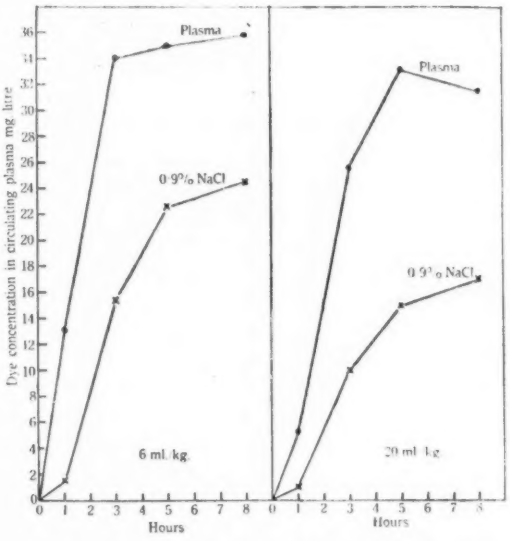

Fig. 1. The concentration of dye in the circulating plasma at intervals after the intraperitoneal injection of 4 mg./kg. dye in 6 or 20 ml./kg. heparinized plasma and 0·9 p.c. NaCl in the rabbit.

Since electrolytes can be absorbed by the blood capillaries and proteins only by the lymph capillaries, the protein concentration of the injected fluid was determined at intervals to see whether the protein level of the plasma altered or whether protein diffused into the injected 0·9 p.c. NaCl solution. These experiments were performed in groups of both rabbits and guinea-pigs after the intraperitoneal injection of 20 ml./kg. of plasma and of 0·9 p.c. NaCl.

The dye in the plasma and remaining peritoneal fluid was estimated in the same manner as in the cat experiments (Courtice and Steinbeck, 1950). The protein and non-protein nitrogen concentrations of the various fluids were determined by micro-Kjeldahl digestion and nesslerization. The colour density was estimated with the Spekker Photoelectric Absorptiometer, using an Iford 603 and Calorex heat filter. For the non-protein nitrogen estimation the proteins were precipitated with trichloracetic acid. The albumin fraction was estimated after precipitation of the globulins and fibrinogen with 27 p.c. sodium sulphite (Steinbeck, 1949).

RESULTS.

Experiments with Rabbits.

The volume of fluid present in the peritoneal cavity of normal rabbits was estimated in a group of 16 animals. Of these the peritoneal cavity contained no free fluid in 9, 0·2 ml. in each of 2 and 1·0, 1·5, 3·7, 25·0 and 35·0 in each of the remainder. In the majority of experiments, therefore, the volume of fluid in the peritoneal cavity before the introduction of plasma or saline must have been either nil or an insignificant fraction of the amount injected. In a few cases, however, a relatively large volume of pre-existing peritoneal fluid was probably present.

In two series of experiments, 6 ml./kg. and 20 ml./kg. of dyed fluid were injected. Blood samples were taken at 1, 3, 5 and 8 hours after the injection for the estimation of plasma dye concentration, while in a few cases samples were taken at 24 and 48 hours. The average results of these experiments are shown in Fig. 1. It is evident from these results that the rise in plasma dye concentration is very much greater after the intraperitoneal injection of dyed plasma than of dyed saline, and the absorption of dye is greater when less fluid is introduced. This overall picture resembles that of the absorption of similar fluids from the pleural cavities, but is in marked contrast to that of the absorption from the alveoli (Courtice and Simmonds, 1949 a, b).

The rise in plasma dye concentration after the introduction of dyed plasma should give a good indication of the rate of absorption of the protein (Courtice and Steinbeck, 1950) by the lymphatics since it appears that the dye-protein complex is absorbed as such. With dyed saline, however, it is possible that the dye may become attached to tissue proteins during the absorption process, so that the dye level in the plasma may not give a true indication of the absorption of this fluid.

TABLE 1.

The rate of absorption in rabbits of heparinized plasma and 0·9 p.c. NaCl after the intraperitoneal injection of 6 ml./kg. fluid to which 4 mg./kg. dye has been added. Expressed as the percentage of the injected volume absorbed.

	Plasma				0·9 p.c. NaCl			
	3 hours	5 hours	8 hours	24 hours	3 hours	5 hours	8 hours	24 hours
	75·6	73·2	100	100	17·1	26·7	75·8	100
	86·4	85·4		100	80·5	58·6	91·5	
	99·8	100·0			35·3	60·6	97·3	
	63·3	94·4				57·9	42·9	
		27·7				47·2		
		73·7						
		100·0						
		100·0						
		100·0						
		100·0						
		100·0						
		100·0						
		100·0						
Mean	81·3	88·8	100	100	44·3	50·2	76·9	100

The actual rates of absorption of plasma and of 0·9 p.c. NaCl were determined by killing groups of animals at definite time intervals and measuring the amount of fluid remaining in the peritoneal cavity. The dye concentration of the remaining fluid was also estimated. In

this way the absorption of fluid and of dye was determined. The results of these experiments are given in Tables 1 and 2. It is evident that plasma is more rapidly absorbed than 0·9 p.c. NaCl in both series of experiments after the introduction of a relatively small volume of fluid, 6 ml./kg., and of a much larger volume, 20 ml./kg. The smaller volume of plasma is all absorbed in 5 hours in the majority of cases and the larger volume in 8 hours. In rabbits, therefore, an appreciable quantity of plasma, half the normal plasma volume, is to a large extent absorbed in 3 hours and almost completely in 8 hours. Isotonic sodium chloride solution, on the other hand, is more slowly absorbed.

TABLE 2.

The rate of absorption in rabbits of heparinized plasma and of 0·9 p.c. NaCl after the intraperitoneal injection of 20 ml./kg. fluid to which 4 mg./kg. dye has been added. Expressed as the percentage of the injected volume and of dye absorbed.

	Time after injection					
	3 hours		5 hours		8 hours	
	Dye	Fluid	Dye	Fluid	Dye	Fluid
Plasma	90·7	85·4	75·0	61·6	100	100
	50·0	23·8	93·0	80·3	100	100
	76·2	74·9	98·8	96·7	100	100
	98·2	97·7			93·0	87·0
					100	100
					100	98·3
					—	92·4
					—	89·2
Mean	78·8	70·5	88·9	79·5	99·0	95·9
0·9 p.c. NaCl	13·0	37·8	79·0	85·0	87·8	86·5
			58·8	50·5	87·8	84·0
			43·4	42·6	69·3	53·5
			32·6	19·8	73·6	69·1
			71·6	69·0	89·0	85·8
			51·0	58·4	66·6	47·3
Mean			56·1	54·2	79·0	70·9

Experiments with Guinea-Pigs.

The amount of fluid normally present in the peritoneal cavity of guinea-pigs was determined in a group of 14 animals. The volume varied from nil to 2 ml., but in the majority of cases was less than 1 ml. Therefore, as in rabbits, the volume of free fluid present in the peritoneal cavity before the injection of plasma or saline must have been nil or a small fraction of the amount introduced, 20 ml./kg.

The rates of absorption of plasma and of 0·9 p.c. NaCl solution from the peritoneal cavity of guinea-pigs are shown in Table 3. It appears that in the guinea-pig the absorption of both plasma and 0·9 p.c. NaCl is somewhat slower than in the rabbit. Whereas 20 ml./kg. plasma is all absorbed in the rabbit in about 8 hours, in the guinea-pig it takes between 16 and 24 hours to absorb the equivalent amount, and the saline is absorbed at about the same rate as plasma. The rise in dye concentration in the plasma, however, is much less after saline

injection than after plasma, as it is in the rabbit. This suggests that when the dye is not tagged on to protein before injection, some of the dye probably combines with tissue protein or is taken up by reticulo-endothelial cells during its absorption.

TABLE 3.

The rate of absorption in guinea-pigs of plasma and of 0·9 p.c. NaCl after the intraperitoneal injection of 20 ml./kg. fluid to which 4 mg./kg. dye has been added. Absorption rates expressed as the percentage of the injected volume absorbed. Dye concentration in plasma in mg./litre.

	Plasma				0·9 p.c. NaCl			
	5 hours	8 hours	16 hours	24 hours	5 hours	8 hours	16 hours	24 hours
	73·0	82·5	74·7	100	52·1	49·3	100·0	100
	55·6	92·3	75·7	100	69·0	59·7	77·5	100
	62·5	75·5	98·5	100	58·1	37·9	71·6	100
	80·2	61·7	82·4	100	51·5	63·0	100·0	100
	38·4	79·0	89·0	100	54·0	39·0	100·0	100
	15·5	50·0		100	59·7	34·2		100
	29·2	31·8		100	45·0	56·5		100
	36·9	82·2		100	24·5			100
	38·6	53·3			43·2			100
	31·6	52·6						
Mean	46·2	66·1	84·1	100	50·8	48·5	89·8	100
Mean final plasma dye concentration:	19·2	22·0	20·6	22·0	9·3	8·0	16·4	28·1

The Protein Content of the Peritoneal Fluid at Various Stages after its Introduction.

When fluid is removed from the peritoneal cavity after the intraperitoneal injection of 0·9 p.c. NaCl, it clots on standing, indicating that fibrinogen has been added. Since protein is absorbed only by the lymphatics, whereas non-protein fluid can be absorbed by the blood capillaries, the pathway of absorption of 0·9 p.c. NaCl would vary with the amount of protein which diffused into this fluid after its introduction into the peritoneal cavity. It was, therefore, decided to estimate the protein content of the remaining peritoneal fluid at various intervals after the introduction of 20 ml./kg. of 0·9 p.c. NaCl. The changes in the protein concentration of the peritoneal fluid after the introduction of plasma were also determined.

In rabbits, the remaining fluid was withdrawn from the peritoneal cavity 1 minute, 3, 5 and 8 hours after introduction. To prevent coagulation heparin was added to the fluid taken from those animals in which 0·9 p.c. NaCl was injected. The results of these experiments are given in Table 4. When plasma is introduced into the peritoneal cavity and withdrawn within 1 minute there may be either no change in protein concentration or a slight fall due probably to the dilution of some existing fluid containing less protein. At 3 and 5 hours the concentration of protein falls, the fall being due to a decrease in the albumin fraction. Since, however, in these experiments nearly all the plasma had been absorbed by this time, there is no definite evidence that albumin is much more rapidly absorbed than globulin.

After the injection of 0·9 p.c. NaCl, protein is added to the fluid. When the fluid is removed within 1 minute, there is a small amount of protein present and the concentration increases to 1½ to 2 p.c. The non-protein nitrogen level of the injected fluid rapidly attains the level in the circulating blood plasma.

TABLE 4.

The protein and non-protein nitrogen content of circulating plasma, injected fluid and fluid remaining in peritoneal cavity at end of experiment. Protein in gm. p.c.; N.P.N. mg. p.c.

Rabbits

Time	Circulating plasma			Fluid injected			Fluid removed		
	N.P.N.	Protein	Alb.	N.P.N.	Protein	Alb.	N.P.N.	Protein	Alb.
Plasma									
1 min.	60	7·13	4·90	47	6·83	4·66	54	6·65	4·24
1 min.	69	6·82	3·93	47	6·83	4·66	57	6·90	4·45
1 min.	29	6·58	3·93	54	6·85	4·47	45	5·75	3·52
1 min.	30	6·07	4·08	54	6·85	4·47	48	5·95	3·80
3 hours	61	8·45	3·80	45	7·35	4·38	68	6·13	1·55
3 hours	53	6·05	5·25	45	7·35	4·38	58	4·56	1·57
5 hours	37	6·27	4·42	54	6·85	4·47	41	4·43	2·57
5 hours	34	5·95	3·72	54	6·85	4·47	37	4·07	2·09
0·9 p.c. NaCl									
1 min.	30	6·28	—				25	0·22	—
1 min.	30	6·06	—				28	0·26	—
3 hours	39	6·20	—				37	1·33	—
3 hours	34	6·55	—				26	1·41	—
5 hours	43	6·65	—				33	1·76	—
5 hours	55	6·53	—				46	1·59	—
5 hours	34	4·80	3·58				34	1·43	1·02
8 hours	35	6·03	—				34	2·13	—
8 hours	29	5·70	—				27	1·61	—

TABLE 5.

The protein and non-protein nitrogen content of the circulating plasma, injected fluid and fluid remaining in the peritoneal cavity at end of experiment. Protein in gm. p.c.; N.P.N. mg. p.c.

Guinea-pigs

Time	Circulating plasma			Fluid injected			Fluid removed		
	N.P.N.	Protein	Alb.	N.P.N.	Protein	Alb.	N.P.N.	Protein	Alb.
Plasma									
3 hours	57	5·60	3·00	55	4·87	2·82	55	4·69	2·50
3 hours	53	5·13	2·89	55	4·87	2·82	50	4·56	2·50
5 hours	44	5·87	3·22	55	4·87	2·82	54	4·81	2·06
5 hours	56	5·69	3·07	55	4·87	2·82	57	3·75	1·87
8 hours	54	6·20	2·91	55	4·87	2·82	50	4·69	2·44
8 hours	57	6·00	3·10	55	4·87	2·82	46	4·06	1·97
0·9 p.c. NaCl									
2 min.	54	4·90	—				22	0·41	—
2 min.	50	4·80	—				22	0·35	—
3 hours	53	5·20	—				45	0·57	—
3 hours	57	4·09	—				48	0·77	—
5 hours	59	5·57	—				57	0·62	—
5 hours	54	4·85	—				48	1·10	—
8 hours	56	4·97	—				34	0·97	—
8 hours	57	4·40	—				55	1·03	—
16 hours	54	5·25	3·52				54	2·44	1·69
16 hours	56	4·77	3·57				56	2·75	1·94

TABLE 6.

The amount of total protein, albumin and globulin in gm./kg. body weight injected intraperitoneally and absorbed in rabbits and guinea-pigs after the intraperitoneal injection of 20 ml./kg. heparinized plasma. Average results for two animals in each experiment.

	Rabbits		Guinea-pigs	
	Injected	Absorbed	Injected	Absorbed
3 hours				
Total	1·47	1·30	0·97	0·31
Albumin	0·88	0·83	0·56	0·21
Globulin	0·59	0·47	0·41	0·10
5 hours				
Total	1·37	1·29	0·97	0·43
Albumin	0·89	0·84	0·56	0·31
Globulin	0·48	0·45	0·41	0·12
8 hours				
Total	—	—	0·97	0·56
Albumin	—	—	0·56	0·35
Globulin	—	—	0·41	0·21

In guinea-pigs the protein changes in the injected fluid at various times after injection are shown in Table 5. When plasma is injected there is a slight fall in the concentration of albumin. After the introduction of 0·9 p.c. NaCl protein diffuses into the fluid until at 16 hours it reaches a level of about 2·5 p.c. As with rabbits the non-protein nitrogen concentration of the injected fluid rapidly attains that in the circulating blood plasma.

The results given in Tables 4 and 5 indicate that the albumin concentration after injection of the plasma falls more than that of globulin. When the volume of fluid absorbed is considered, however, it is evident that globulin is absorbed nearly as rapidly as albumin. In Table 6 are given the amounts of total protein, albumin and globulin injected and absorbed in groups of rabbits and guinea-pigs killed at various intervals after injection.

General Post-Mortem *Appearance.*

(i) *After injection of plasma.* In the series of cats (Courtice and Steinbeck, 1950) as well as in this much larger series of rabbits and guinea-pigs the macroscopic *post-mortem* appearance after an intraperitoneal injection of dyed plasma is similar. In the peritoneal cavity the diaphragmatic peritoneum is deeply stained over the central tendon and in tracts radially arranged with this area and the periphery. Staining may also be intense at the parieto-phrenic boundary. On the other hand, the parietal and visceral peritoneum in the majority of cases is only lightly stained, except where pockets of dyed-plasma have remained, e.g. in relation to the upper poles of the kidney or along the paracolic gutters, and along the lines of the intercostal spaces laterally. The greater omentum is generally lightly stained but more definitely along the adiposo-vascular arcades. In some cases dyed fluid was seen between the layers of the mesentery and less often a definite collection was observed at the base. In only two cats were the inguinal lymph nodes stained, but the blue colour was not nearly as intense as that of the anterior mediastinal group of nodes. From this general appearance, therefore, it seems that most of the dyed plasma passes through the diaphragmatic peritoneum, but some is also absorbed through the visceral and parietal peritoneum and through the omentum.

When the thoracic cavity is opened, the diaphragm is seen to be deeply stained. About the central tendon is a boundary lymphatic into which smaller channels appear to radiate. This lymph vessel connects with the parasternal lymphatics anteriorly and with a posterior group near the fleshy crus of the diaphragm. These appear to extend cephalically and in only one case was a vessel noted passing to the lumbar region. Around the periphery of the diaphragm there is another channel connecting with the parasternal and crural group of lymphatics and directly or indirectly with the vessel about the central tendon. Apparently connecting the two boundary lymphatics are those in the fleshy mass of the diaphragm running radially. About these the dye in some cases has diffused. These boundary vessels appear to lie immediately below the pleura and are distended compared with those in normal animals.

TABLE 7.

(a) The concentration of dye in the circulating plasma, pleural and remaining peritoneal fluid at intervals after the intraperitoneal injection of 4 mg./kg. dye in plasma.

	Fluid injected ml./kg.	Time after injection hr.	Pleural fluid ml.	Remaining peritoneal fluid ml.	Concentration of dye mg./l.		
					Circulating plasma	Pleural fluid	Peritoneal fluid
Rabbits	6	5	1·2	0·5	38·5	175·0	137·5
	6	5	1·5	5·0	14·9	50·0	198·5
	6	5	1·0	5·5	27·3	89·0	214·0
	6	5	0·2	2·0	36·5	106·5	167·5
	6	5	0·4	0·0	39·0	167·5	—
	20	8	3·0	3·7	29·2	99·0	117·0
	20	3	0·7	0·5	27·6	150·0	150·0
	40	8	3·5	25·5	14·9	74·0	82·5
Guinea-pigs	20	5	2·8	2·5	24·8	187·5	195·0
	20	8	0·7	1·4	21·6	144·0	161·0
	20	5	0·4	8·0	14·6	70·0	163·0
	20	5	0·6	6·8	13·2	73·0	151·0
	20	8	1·4	2·4	23·6	121·0	144·0
	20	8	1·4	7·1	18·6	104·0	148·0
	20	8	2·0	10·1	15·8	84·0	139·0
	20	16	3·3	1·5	17·6	104·0	122·0

(b) Pleural fluid in 5 rats 24 hours after intraperitoneal injection of 50 ml./kg. plasma.

ml. injected	ml. recovered	
	Peritoneal cavity	Pleural cavity
10·4	0·0	2·2
10·3	0·0	3·2
10·7	3·0	4·2
7·6	0·0	4·3
6·8	0·0	3·5

The middle mediastinum as it lies over the central tendon often contains dyed fluid which is partly free within the tissues. Such an appearance is also frequently noted in the anterior mediastinum and may spread widely into the anterior thoracic wall. In some rabbits after an intraperitoneal injection of 20 ml./kg. dyed plasma, 0·2–0·3 ml. of blue fluid could be collected from this region. The pleural cavities of rabbits and guinea-pigs often contained free

fluid which was only rarely present in normal animals. In a small series of rats in which 50 ml./kg. rabbit plasma were injected intraperitoneally, the plasma was practically all absorbed from the peritoneal cavity, but a considerable amount of the fluid was found at 24 hours in the pleural cavities. The pleural fluid was quite blue, so the concentration of dye was estimated in this as well as in the plasma and the remaining peritoneal fluid. The results are given in Table 7. It is evident that the volume of pleural fluid may be appreciable and that the dye concentration is always much greater than that in the plasma, but somewhat less than that in the remaining peritoneal cavity. It appears, therefore, that this fluid has not come from the pleural blood capillaries, but from the lymphatics of the anterior mediastinum during the passage of the absorbed fluid from the diaphragmatic lymph vessels through the mediastinum. This pleural fluid will be later absorbed as described by Courtice and Simmonds (1949, b), and in this process the parietal pleural lymphatics will appear blue especially along the costal margins.

The main lymph channels from the diaphragm are the parasternal, passing retrosternally in relation to the internal mammary arteries and veins. These vessels, between which no major anastomosis was seen, drained into the groups of lymph nodes found in the superior mediastinum, dorso-superior to the thymus and those more laterally toward the apex of the pleura. From these nodes which were deeply blue, efferents passed as a rule to the right lymph duct. In a few instances in the cat two crural channels were observed just anterior to the vertebrae, while in one cat and two rabbits the pulmonary hilar lymph nodes on the right side were blue, but this was unusual.

(ii) *After injection of 0·9 p.c. saline.* The remaining peritoneal fluid was always collected by pasteur pipette into a graduated tube. On exposure in such a tube this fluid clotted uniformly. Before and during the withdrawal of this fluid fibrin plaques were noted either free within the fluid or loosely attached to the peritoneum and mesentery. These plaques were firm, whitely opaque, generally comma-shaped, always multiple and some were large. With the longer preparations the fluid remnant was opaque as the result of contained cells and debris.

The overall appearance of the peritoneum was similar to that in the plasma experiments, the difference being mainly of degree. However, the general peritoneum in the guinea-pig experiments showed some staining which was maximal and readily apparent in the 16-hour preparations. This dyeing of the peritoneum also occurred in plasma-injected pigs at the same time. The ventral membrane, after the diaphragmatic, was most heavily stained. The duration of contact with the dye containing fluid appears to be the factor in this staining which is additional to, rather than incidental to, absorption, as it does not occur with the more rapid absorption in rabbits.

Some rabbits, and fewer guinea-pigs, had retroperitoneal collections of dyed fluid tracking toward the diaphragm. The appearance was of fluid passing into the tissues and being retained as a gel or a semi-gel. An explanation is not at present apparent. The pathways of dye distribution were identical with the plasma experiments. In the rabbit these pathways were always less blue initially than after plasma injections. This confirms for these experiments the lower plasma T-1824 concentration. In some animals—8 hours in the rabbits and 16 hours in the pigs—there was diffusion of dye about the parasternal lymphatics though never approaching the extent of diffusion after plasma injections. This diffusion was at no time associated with a collection of pleural fluid, in these animals nor in a group of rats in which 50 ml./kg. was introduced intraperitoneally.

DISCUSSION.

The present experiments have dealt mainly with the rate of absorption of two fluids, crystalloid and protein-rich in nature, from the peritoneal cavity of rabbits and guinea-pigs. The most striking feature of the experiments is the rapid absorption of heparinized plasma by the diaphragmatic lymphatics and its ultimate passage along the parasternal lymph channels into the blood stream. In the rabbit this absorption of plasma is greater than that of an equivalent amount of 0·9 p.c. NaCl, while in the guinea-pig the rates of absorption of both fluids are somewhat the same and slower than in the rabbit. Assuming a plasma volume of 40 ml./kg. body weight for the rabbit (Courtice and Gunton, 1949), this animal can absorb from the peritoneal cavity a volume of plasma equal to half the plasma volume in 8 hours, whereas the guinea-pig can absorb an equivalent volume in 16–24 hours.

Why a crystalloid solution, 0·9 p.c. NaCl, is not absorbed more rapidly than plasma in these animals is not known. The pathway of absorption of plasma is only the lymph capillaries and the large protein molecules have first to pass through the peritoneum, although it appears that the proteins can readily penetrate the diaphragmatic peritoneum. A crystalloid solution on the other hand, once it has passed through the peritoneal lining should be absorbed by both the blood and the lymph capillaries, and so would be expected to be absorbed more rapidly than plasma. It would appear that the lymphatics do not so readily take up the crystalloid solution as they do plasma, although there may be an excess of free fluid in the peritoneal cavity ready to be absorbed. After an intraperitoneal injection of plasma there is often free fluid in the pleural cavity and in the anterior mediastinal tissues, but this is not seen after the intraperitoneal injection of 0·9 p.c. NaCl. This suggests that the lymph nodes in the anterior mediastinum act as a partial obstruction to the free flow of lymph. The plasma rapidly enters the diaphragmatic lymphatics and is forced along the lymph channels by respiratory movement. If these lymph channels are dilated and the nodes act as a partial obstruction, some of the lymph passes back into the mediastinal tissues, through the pleura and into the pleural cavities. The high protein content of this pleural fluid and the high dye concentration indicate that it comes from the lymph vessels draining the peritoneal cavity as they pass through the thoracic cavity. The fact that no pleural fluid accumulates even after large injections of 0·9 p.c. NaCl (50 ml./kg.) suggests that this crystalloid fluid passes less readily into the diaphragmatic lymphatics than does plasma.

The absorption of plasma from the peritoneal cavity appears to be straightforward. The protein content of the injected fluid may fall somewhat after a few hours, but it seems probable that there is little alteration in its composition while it remains in the peritoneal cavity awaiting absorption. When

0·9 p.c. NaCl is injected, however, there is an exchange of substances and probably the composition of the fluid rapidly assumes that of the tissue fluid in the peritoneum. The non-protein nitrogen level very rapidly rises to that of the blood and the protein concentration rises to about 1·5 p.c., which is what Maurer, Warren and Drinker (1940) found in the free peritoneal fluid of normal rabbits. The concentrations of other substances have not been determined, but it is probable that the composition of the injected saline rapidly approaches that of the tissue fluid of the peritoneum. The absorption of 0·9 p.c. NaCl is, therefore, more complicated than that of plasma. During this early stage of exchange, the water exchange is not known, although there is no increase in the volume at 3 hours. After this equilibrium has been reached the absorption should be more rapid than plasma, because the proteins can be absorbed by the lymph vessels and the non-protein fluid by the blood capillaries, as in the absorption of tissue fluid from any region. The lymphatics, however, do not seem to take up this low-protein fluid as readily as they do a protein-rich fluid, plasma, although the physical forces produced by respiratory movement should be the same in each case.

Compared with the absorption from the lung alveoli and pleural cavities (Courtice and Phipps, 1946; Courtice and Simmonds, 1949 a, b) the absorption of plasma from the peritoneal cavity is rapid. When approximately the same volume of heparinized plasma, 6 ml./kg., is introduced into these three regions, it is absorbed in 5 hours from the peritoneal cavity, 24 hours from the pleural cavity and 4 days from the lung alveoli. In all cases the protein passes along the same final pathways, the majority by the right lymph duct and the remainder by the thoracic duct, and in each instance respiratory movement affects the flow of lymph. It would, therefore, appear that it is the passage of the protein-rich fluid through the lining membrane before reaching the lymph capillaries that is the most important factor in absorption. In the lung alveoli the surface area is large and water and 0·9 p.c. NaCl are very rapidly absorbed directly into the blood stream, but when plasma proteins are added the absorption is greatly slowed down. This indicates that the protein molecules can pass but slowly through the alveolar membrane. In the pleural cavity absorption of the proteins through the pleural membrane into the pleural and mediastinal lymphatics is more rapid, while in the peritoneal cavity the diaphragmatic peritoneum, although small in area compared with the rest of the peritoneum, the pleura or the lung alveolar membrane, allows ready access of the protein molecules into the underlying diaphragmatic lymph capillaries. Once the protein enters the mediastinal lymphatics, the same forces, those due to respiratory movement, are responsible for the propulsion of the lymph through the lymph channels ultimately to the blood stream.

Whether the rates of absorption of plasma and of saline from the peritoneal

cavity of other animals are similar to those of the rabbit and guinea-pig is at present under investigation. In man there are more lymph nodes than in animals and maybe these will slow down the ultimate absorption of plasma by the lymphatics.

SUMMARY.

The rates of absorption of heparinized plasma and of 0·9 p.c. NaCl introduced into the peritoneal cavity of rabbits and guinea-pigs have been investigated.

In the rabbit plasma is absorbed more rapidly than 0·9 p.c. NaCl when 6 and 20 ml./kg. of fluid are injected. A rabbit can absorb half its normal plasma volume (20 ml./kg.) in 8 hours.

The absorption of these two fluids from the peritoneal cavity of the guinea-pig is somewhat slower than in the rabbit, and plasma and 0·9 p.c. NaCl are absorbed at approximately equal rates when 20 ml./kg. is introduced.

The plasma is absorbed by the lymphatics mainly through the diaphragmatic peritoneum, and in its passage through the parasternal lymphatics may diffuse into the mediastinal tissues and then into the pleural cavities. When 0·9 p.c. NaCl is injected there is at first an exchange of substances, so that the fluid resembles tissue fluid with about 1·5 p.c. protein. This is then absorbed partly by the lymphatics and partly by the blood capillaries.

Acknowledgment. We should like to thank Mr. J. Harding for technical assistance in these experiments.

REFERENCES.

Bolton, C. (1921): J. Path. Bact., 24, p. 429.
Courtice, F. C. and Gunton, R. W. (1949): J. Physiol., 108, p. 405.
Courtice, F. C. and Simmonds, W. J. (1949 a): *Ibid.*, 109, p. 103.
Courtice, F. C. and Simmonds, W. J. (1949 b): *Ibid.*, 109, p. 117.
Courtice, F. C. and Steinbeck, A. W. (1950): Austral. J. exp. Biol., 28, p. 161.
Maurer, F. W., Warren, M. F. and Drinker, C. K. (1940): Amer. J. Physiol., 129, p. 635.
Steinbeck, A. W. (1949): Austral. J. exp. Biol., 27, p. 55.
Watkins, A. L. and Fulton, M. N. (1938): Amer. J. Physiol., 122, p. 281.

MIXED BACTERIAL GROWTH

I. ENUMERATION OF THE VIABLE ORGANISMS IN MIXED POPULATIONS OF A VIRIDANS STREPTOCOCCUS AND *STAPHYLOCOCCUS ALBUS*

by D. I. ANNEAR AND LILA HAYES

(From the Institute of Epidemiology and Preventive Medicine, Prince Henry Hospital, Sydney).

(Accepted for publication 4th October, 1949.)

Bacteria occur naturally in mixed populations rather than in pure culture and, as Woods (1947) pointed out, information on their behaviour under these conditions is much needed. The identification and enumeration of the component strains of mixed bacterial flora are important aspects of such studies.

It appears that no methods other than those relying upon differences in colonial characteristics have been applied to the problem of enumerating the individual strains of viable organisms in mixed cultures. Considering the simplest case, that of two organisms, provided that the numerical ratio of the strains in the mixture was not too high and that the colonies of the strains were readily distinguishable on the recovery medium used, these methods would appear to be satisfactory. The limiting ratios that can be established by such methods, however, place a severe restriction on their usefulness in studies of quantitative relationships in bacterial associations.

It was felt that selective agar media might provide a more satisfactory method for obtaining estimates of the component strains of mixed bacterial cultures and enable the enumeration of each organism to be made when it was heavily outnumbered by the other.

In preliminary experiments with a number of antibacterial substances, it was found that in nutrient agar, crystal violet (CV) had a greater inhibitory effect on strains of *Staph. albus* than on strains of viridans streptococci whereas the opposite effects were observed with sulphanilamide (SA). A strain of each of these organisms was selected for study.

It was clear that before the selective media could be regarded as dependable for estimating the viable organisms in mixed cultures of unknown composition the following points needed investigation with populations of known numbers.

1. The selection of a concentration of CV which would inhibit the staphylococcus but permit maximal recovery of the streptococcus.
2. The selection of a concentration of SA which would inhibit the streptococcus but permit maximal recovery of the staphylococcus.
3. Determination, in the selected concentration of CV, of the number of staphylococci which could be inhibited and the effect of the presence of such numbers of staphylococci on the recovery of the streptococcus.
4. Determination, in the selected concentration of SA, of the number of streptococci which could be inhibited and the effect of the presence of such numbers of streptococci on the recovery of the staphylococcus.

5. The comparison of the total count in mixtures of the two organisms, as revealed in nutrient agar, with the sum of the individual estimates as derived from the counts in the selective agars.

The earlier part of this paper is concerned with these points and the latter with the application of the principles to the study of some dynamic associations of the two organisms.

Statistical Control.

Fisher, Thornton and Mackenzie (1922) showed that the frequencies of constant volume samples of thin bacterial suspensions conform under ideal conditions to a Poisson series, as do the viable counts from such suspensions. The variance of a Poisson population is equal to the mean, the square root of which is the standard deviation. The sampling error of the ideal colony count is therefore equal to the square root of the number of colonies counted.

In this investigation viable counts were made with the organisms in a variety of circumstances and it was considered that testing the replicate counts for conformation to Poisson distribution would provide useful evidence for assessing the validity of the method and results.

Evidence that replicate colony counts are distributed as variates of a Poisson distribution may be obtained by use of the statistic χ^2, ($\chi^2 = \Sigma(x - \bar{x})^2/\bar{x}$). When sufficient sets of replicate colony counts are available, conformation to the Poisson series may be tested for, either by adding the individual χ^2 values and testing the total with the appropriate number of degrees of freedom or by comparing the observed distribution of χ^2 values with the expected and determining the Goodness of Fit. The former method is the only practicable one for a small number of sets. As in this work the number of replicates per set varied from 2 to 5, it was more convenient to assign the χ^2 values to probability (P) class intervals before testing the frequency distributions for Goodness of Fit.

Treatment effects in this work were also assessed by application of the statistic χ^2. This involved resolving the total χ^2 from samples as a whole into *within* and *between* treatment components. If replicate inocula are sown in two or more different media and each medium allows ideal colony development, the variance of the counts both *within* and *between* each medium will be that of a Poisson distribution. The procedure by which the variances were tested is best explained by a hypothetical case. Supposing, for example, replicate inocula sown into two different media, "A" and "B", resulted in colony counts of 15, 20, 22 and 14, 15, 19 respectively, the procedure for analysis would be as follows:

	Treatment A		Treatment B	
	x	x^2	x	x^2
	15	225	14	196
	20	400	15	225
	22	484	19	361
	57	1,109	48	782
$\bar{x}$ =	19		16	
$\frac{T^2}{N}$ =	$\frac{57^2}{3}$ =	1,083	$\frac{48^2}{3}$ =	768
$\Sigma(x - \bar{x})^2$ =		26		14

Grand total $= 105$

Grand mean $= \frac{57 + 48}{6} = 17{\cdot}5$

Correction factor $= \frac{1052}{6} = 1{,}838$

	Sums of squares	Mean	χ^2	D.f.	P*
Treatment A	26	19	1·368	2	·50
Treatment B	14	16	0·875	2	·63
Within treatments	26 + 14 = 40	17·5	2·286	4	·68
Between treatments	1,083 + 768 − 1,838 = 13	17·5	0·743	1	·40
Total	1,109 + 782 − 1,838 = 53	17·5	3·029	5	·70

* Here as everywhere in this paper P = probability of a higher value of χ^2 than the observed occurring by chance.

In this hypothetical case the P values are such as to suggest that the variations of the counts both *within* and *between* each treatment were chance ones only. In assessing the variances of treatments, the range $P = \cdot01$–$P = \cdot99$ was usually regarded as indicative of non significance. However, as there were for most treatments a large number of observations, judgment of any particular treatment or series of treatments was modified by that of the group as a whole. Where large numbers of independent comparisons were available, frequency distributions of the P values were compiled, while for small numbers the summation method was used.

General Methods.

Media.

The broth employed was a beef heart infusion fortified with Neopeptone (1·0 p.c.) and sodium chloride (0·5 p.c.). The nutrient agar medium had the same composition plus agar (Davis Gelatine Pty. Ltd., 1·5 p.c.). Both broth and agar were used at pH 7·4. The selective agars were prepared by adding aqueous solutions of the drugs to the melted nutrient agar and were sterilized by autoclaving. One quarter strength broth was used to dilute the bacterial suspensions.

Bacterial Inocula.

The bacterial inocula, both for the investigations on populations of known numbers and for the growth curve experiments were obtained from 20 hour broth cultures.

Viable Counts.

Ehrlenmeyer flasks of 200 ml. capacity were used instead of plates for making viable counts. With a wide mouthed pipette 10 ml. of agar were introduced into each flask, this quantity being sufficient to give a depth of approximately 5 mm. of agar. The inoculum invariably contained in 1 ml. of diluent, was introduced into the melted agar maintained at 45° C. The flasks, after one gentle rotation, were cooled in a shallow layer of water where the agar set rapidly. Counts were made after the flasks had been incubated at 37° C. for 2 days. The colonies could be counted satisfactorily in daylight by resting the inverted flask on the mouth of a tin, the diameter of which was somewhat less than that of the flask and the inside of which was painted black.

The flasks had several advantages over plates, the chief one being that in them the organisms were distributed more easily in the agar. This was due to the method of inoculation, a feature which also appeared to lessen the chances of the development and spreading of colonies on the agar-glass interfaces. Contamination was negligible in the cotton wool plugged flask.

EXPERIMENTAL.

STATIC POPULATIONS.

Titrations of Selective Drugs.

As an initial step in the development of the method, experiments were carried out to determine the tolerance in nutrient agar of the streptococcus to CV and of the staphylococcus to SA. It was not necessary for the purposes of this work to determine with accuracy the lowest concentration of drug inhibitory to the organism, but only to establish below this critical level, a concentration which could be relied upon to permit maximal recovery of the organism.

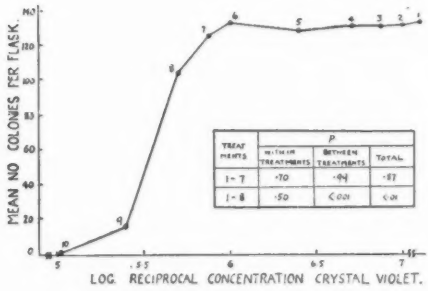

Fig. 1. Effect of crystal violet in nutrient agar on the colony counts of a viridans streptococcus. The medium for treatment 1 was nutrient agar. Means are from 5 flask counts.

Inset: Probability (P) values from a χ^2 analysis of the counts.

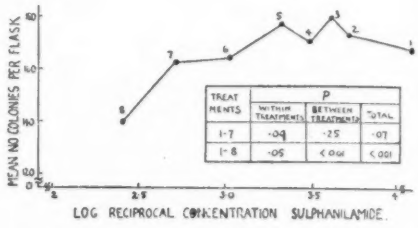

Fig. 2. Effect of sulphanilamide in nutrient agar on the colony counts of *Staph. albus*. The medium for treatment 1 was nutrient agar. Means are from 5 flask counts.

Inset: Probability (P) values from a χ^2 analysis of the counts.

Fig. 1 is a diagrammatical representation of the results from an experiment in which a range of CV concentrations in nutrient agar was tested on replicate countable inocula of the streptococcus, and Fig. 1 inset, the P values from a χ^2 analysis of the counts. Treatments 9 and 10 were excluded from the analysis because of their obvious inhibitory effects on the streptococcus. The analysis showed that there was no significant difference between the counts of treatments 1–7 but that treatment 8 resulted in a significantly lower recovery of the organism. It was evident that at concentrations of CV below 1/750,000 the streptococcus was not inhibited.

In Fig. 2 and its inset are presented the results for a similar experiment in which replicate countable inocula of the staphylococcus were sown in nutrient agar containing a range of concentrations of SA. The analysis showed that there was no significant difference between the counts of treatments 1–7 but that treatment 8 resulted in a significantly lower recovery of the staphylococcus. It was evident that at concentrations of SA below 1/500, the staphylococcus was not inhibited.

The experiments shown in Figs. 1 and 2 were selected from several of similar design and were those experiments in which the lowest drug tolerances were observed.

It was not necessary to determine with accuracy the relationship between CV concentration and staphylococcal recoveries or between SA concentration and streptococcal recoveries. With inocula of approximately 10^5 organisms, it was shown that staphylococcal growth was

completely inhibited by CV, 1/1,000,000 and partially inhibited by CV, 1/10,000,000 and that streptococcal growth was completely inhibited by SA, 1/8,000 and partially inhibited by SA, 1/16,000. Clearly there were significant differences in the sensitivities of the two organisms to CV and SA.

Efficiency of Selective Agars.

Experiments were designed to determine for chosen drug concentrations in nutrient agar, the heaviest inoculum (test dose) of the susceptible organism which could be inhibited and the effect of the presence of such numbers on the recovery of the resistant organism. The concentration of CV selected was 1/1,000,000, a concentration shown to have no inhibitory effect on the recovery of the streptococcus but one which would inhibit large numbers of staphylococci. The SA concentration selected was 1/3,000, a level considerably lower than that shown to be inhibitory to the staphylococcus but one which would inhibit large numbers of streptococci. While concentrations of SA less than 1/500 did not reduce the number of staphylococcal colonies recovered, there was a noticeable diminution in colony size until the drug concentration was reduced to about 1/3,000.

TABLE 1.

(*a*) *Effect in crystal violet* (*CV*) *agar of* Staph. albus *on the colony counts of a viridans streptococcus.*

Staphylococcus (test dose) sown in CV agar (control flasks) and in CV agar containing a countable inoculum of the streptococcus (test flasks). Streptococcus also sown in nutrient agar. Concentration of crystal violet = 1/1,000,000. Means are from 5 flask counts.

Treatment No.	Test dose of Staph.	Mean Strep. count in nutrient agar	Mean count in CV agar		Strain ratio Strep.:Staph. in test flasks
			Test flasks	Control flasks	
1	—	52	—	—	—
2	0		52	—	—
3	$1{\cdot}8 \times 10^5$		53	0	1:3,500
4	$1{\cdot}8 \times 10^6$		52	0	1:35,000
5	$1{\cdot}8 \times 10^7$		53	0	1:350,000
6	$1{\cdot}8 \times 10^8$		47	12	1:3,500,000

(*b*) *Probability* (P) *values of a* χ^2 *analysis of the nutrient agar and test flask counts, the means of which are shown in Table 1 (a).*

Treatments	*P*		
	Within treatments	Between treatments	Total
1–5	·42	·99	·65

Table 1 shows the means of the counts from an experiment in which a range of concentrations of the staphylococcus was sown in CV agar and in CV agar containing a countable number of the streptococcus. The Table also shows the P values from a χ^2 analysis of the counts. In the case of treatment 6, a few colonies appeared in the CV agar flasks inoculated with the staphylococcus alone so the counts from the corresponding test flasks were omitted

from the analysis. The CV agar inhibited the development of as many as $1{\cdot}8 \times 10^7$ staphylococci and the analysis showed no significant difference between the recoveries of the streptococcus in CV agar containing this number of staphylococci, in CV agar alone and in nutrient agar.

TABLE 2.

(a) Effect in sulphanilamide (SA) agar of a viridans streptococcus on the colony counts of Staph. albus.

Streptococcus (test dose) sown in SA agar (control flasks) and in SA agar containing a countable inoculum of the staphylococcus (test flasks). Staphylococcus also sown in nutrient agar. Concentration of SA = 1/3,000. Means are from 5 flask counts.

Treatment No.	Test dose of Strep.	Mean Staph. count in nutrient agar	Mean count in SA agar		Strain ratio Staph.:Strep. in test flasks
			Test flasks	Control flasks	
1	—	66	—	—	—
2	0		64	—	—
3	$1{\cdot}3 \times 10^5$		65	0	1:1,700
4	$1{\cdot}3 \times 10^6$		74	0	1:17,000
5	$6{\cdot}5 \times 10^6$		53	1	1:85,000
6	$1{\cdot}3 \times 10^7$		3	2	1:170,000

(b) Probability (P) *values of a* χ^2 *analysis of the nutrient agar and test flask counts, the means of which are shown in Table 2 (a).*

Treatments	*P*		
	Within treatments	Between treatments	Total
1–4	·51	·15	·37

The results from a similar experiment with SA agar are shown in Table 2. Treatments 5 and 6 were excluded from the χ^2 analysis of the counts because of the colonies which appeared in the control flasks and because of the obvious suppression of the staphylococcal colonies in the test flasks. This end point effect was observed consistently in the case of SA agar. The SA agar inhibited the development of as many as $1{\cdot}3 \times 10^6$ streptococci and there was no significant difference between the recoveries of the staphylococcus in SA agar containing numbers of streptococci as high as this, in SA agar alone and in nutrient agar.

The antibacterial activity of sulphanilamide is known to be influenced by various biological materials and processes. Pike and Foster (1944) demonstrated the ability of SA agar of a concentrated inoculum of bacteria to make possible the growth of a lighter dispersed inoculum of the same or another strain which otherwise would be inhibited. Under the conditions of the experiments in this work the phenomenon was observed occasionally. Where there was a heavy concentration of the streptococcus in the SA agar, prolonged incubation sometimes resulted in the development of satellite colonies of this organism around the larger staphylococcal colonies. Within the standard incubation period of 48 hours, satellitism was rare, and, when it did occur, the satellite colonies were minute and in no way interfered with the staphylococcal colony count.

The experiments shown in Tables 1 and 2 were selected from several of similar design and were those which showed the lowest efficiencies of the selective agars. On the evidence of

these experiments it was considered that the streptococcal estimates were reliably established in CV agar (1/1,000,000) containing as many as 20 million staphylococci and that the staphylococcal estimates were reliably established in SA agar (1/3,000) containing as many as one million streptococci.

Growth Curve Control Experiments.

As the growth curve experiments in this work were spread over several months and different batches of media were used it was considered necessary to check before each growth curve the relationships between the sensitivities of the organisms and the selective media.

TABLE 3.

(a) Growth curve control experiments.

Mean colony counts and test doses from experiments in which a countable inoculum (a) of a viridans streptococcus was sown in nutrient agar (1), in CV agar (2), and in CV agar containing a test dose of *Staph. albus* (3); a countable inoculum (b) of *Staph. albus* was sown in nutrient agar (4), in SA agar (5), and in SA agar containing a test dose of viridans streptococcus (6); (a) and (b) were sown together in nutrient agar (7).

Concentration of SA = 1:3,000. Concentration of CV = 1:1,000,000. Means are from 5-flask counts.

	Mean number of colonies								Test doses	
	Streptococcus (a)			Staphylococcus (b)			Strep. (a) and Staph. (b)	Strep. (1) (2) (3) + Staph. (4) (5) (6)	Staph.	Strep.
Exp.	Nutrient agar (1)	CV agar (2)	CV Agar (Staph. present) (3)	Nutrient agar (4)	SA agar (5)	SA Agar (Strep. present) (6)	Nutrient agar (7)			
1	103	102	102	155	156	146	242	254	$1{\cdot}4 \times 10^7$	$5{\cdot}1 \times 10^6$
2	53	53	60	62	69	59	115	119	$5{\cdot}5 \times 10^6$	$2{\cdot}8 \times 10^6$
3	30	29	30	95	104	95	133	128	$9{\cdot}9 \times 10^6$	$3{\cdot}0 \times 10^6$

Each selective medium was also sown with the test dose of the susceptible organism alone. The counts from these flasks are not shown. They were uniformly nil.

(b) Probability (P) values corresponding to the sums of χ^2 values from an analysis of counts of ten experiments the means of three of which are shown in Table 3 (a).

	Streptococcus			Staphylococcus			Strep. and Staph.	
	Within treatments	Between treatments	Total	Within treatments	Between treatments	Total	Nutrient agar (7)	*
P	·88	·94	·94	·65	·56	·65	·92	·52

*For this column $\chi^2 = \left(\frac{T_1}{15} - \frac{T_2}{5}\right)^2 \Big/ \frac{T_1}{15^2} + \frac{T_2}{5^2}$ where T_1 = total of counts from (1), (2), (3), (4), (5), (6), and T_2 = total of counts from (7) (see text).

The experimental design and the results of three of the ten such experiments done are shown in Table 3. In all experiments the drug concentrations were fixed and the test doses in each arranged to exceed any that were expected to occur in the growth curve contemplated. Included in the experiments was a design for testing the agreement between the counts resulting from the inoculation of the two organisms together in nutrient agar and the sum of those from the individual estimates.

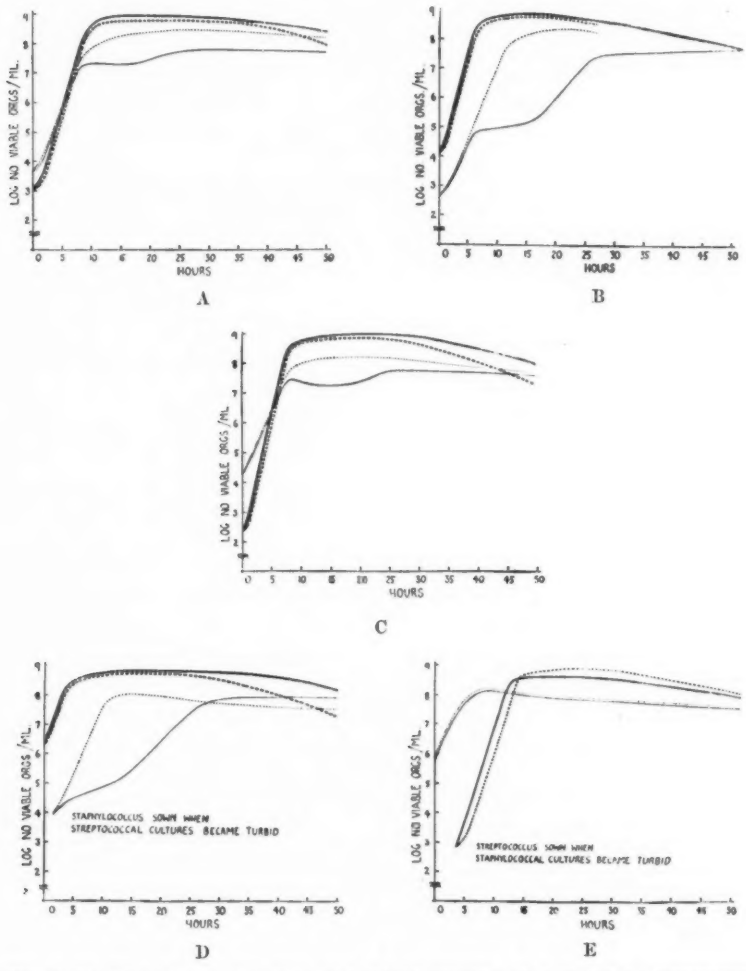

Fig. 3 (A–E). Curves for pure and mixed growths of a viridans streptococcus and *Staph. albus* in nutrient broth at 37° C. Streptococcus (alone) ••••••; streptococcus (mixture) ——; staphylococcus (alone) - - - -; staphylococcus (mixture) ———.

Data from which curves were prepared are shown in Table 4.

In all experiments, each selective agar inhibited all organisms of the susceptible strain inoculated and the analysis showed that for each strain there was no significant difference between the counts in nutrient agar, in the selective agar to which the strain was resistant and in this selective agar containing the stated test dose of the susceptible organism. The analysis also showed that the counts of the two organisms colonizing together in nutrient agar varied as small Poisson samples ($P = \cdot 92$) and that there was no significant difference between these counts and the estimates as derived from the sums of the individual counts ($P = \cdot 52$). In the three experiments shown the test doses of streptococci were slightly higher than the limiting dose ($1 \cdot 3 \times 10^6$) as previously derived (Table 2). The order of the difference was not great and such variation is to be expected with this type of experimental material. The maximum number of streptococci in mixed inocula sown in SA agar flasks during the actual growth curves was in any case below $1 \cdot 3 \times 10^6$.

Growth Curves.

It was considered that the points outlined in the introduction of this paper had been adequately investigated and that the results indicated that the method would be satisfactory in measuring the population changes in associations of the two organisms, provided the limitations of the selective agars were not exceeded.

Experiments were conducted along the usual lines for studying bacterial growth curves and the organisms were cultivated separately as well as in association. The inocula were the only variables studied and were adjusted to provide both high and low initial ratios of the two organisms.

The organisms were grown at 37° C. in 400 ml. of broth contained in 500 ml. cotton wool plugged Florence flasks. Before the 24 hour and subsequent samples were taken, the flasks were removed from the water bath, rotated gently and replaced in the bath. Except for withdrawal of the samples, the flasks were not disturbed during the earlier samplings. Constant check for contamination of the cultures was made with blood agar platings and smears.

Table 4 shows the logarithms of the estimates and the ratios of the organisms growing in pure and associated culture, and Fig. 3 the curves constructed from the data. In Exp. B the 52 hour samples of the pure cultures were contaminated so the counts from these readings were discarded.

From a comparison of the pure culture curves it may be seen that the streptococcus was a faster growing organism, that it reached a higher maximum and that it entered its decline phase and died out more rapidly than the staphylococcus.

In all of the mixed cultures but that of Experiment E the staphylococcus was suppressed for some time during its logarithmic growth phase. Table 5 shows the logarithms of the numbers of each organism in the mixtures at the beginning of the associations and at the approximate time when the staphylococcus began to be suppressed. It seemed clear that while both organisms were in the logarithmic phase, it was not until the streptococcus had reached a high concentration that the staphylococcus was suppressed. In Experiments A and C the degree of suppression of the staphylococcus was greater than in Experiments B and D. The total number of viable organisms present in the mixture at the time of suppression of the staphylococcus was greater in the former two experiments than in the latter two, which suggests that the change in slope was related to the total number of viable cells present. In Experiment E, where the staphylococcus was not inhibited, the initial ratio of the staphylococcus to the streptococcus was very high (42,000:1) and the staphylococcus had almost reached its maximum population before the streptococcus was introduced into the flask. Also

Experiment E was the only instance in which the growth of the streptococcus was influenced by the presence of the staphylococcus during the logarithmic phase. In the presence of the heavy concentration of the staphylococcus a more rapid entry of the streptococcus into its logarithmic phase was initiated. After both organisms in the mixture had passed into the stationary phase, the streptococcus died out more rapidly than the staphylococcus. The curves show also that in the mixtures the streptococcus died out less rapidly than it did in pure culture.

TABLE 4.

Pure and mixed growths of a viridans streptococcus and Staph. albus *in nutrient broth at 37° C.*

Streptococcus in pure culture enumerated from 6 replicate counts (3 in nutrient agar and 3 in crystal violet agar).
Staphylococcus in pure culture enumerated from 6 replicate counts (3 in nutrient agar and 3 in sulphanilamide agar).
Streptococcus in mixed culture enumerated from 3 replicate counts in crystal violet agar.
Staphylococcus in mixed culture enumerated from 3 replicate counts in sulphanilamide agar.
Streptococcus and staphylococcus (collectively) in mixed culture enumerated from 3 replicate counts in nutrient agar (direct) and from the sum of the selective agar counts (calculated).
Concentration of crystal violet = 1/1,000,000. Concentration of sulphanilamide = 1/3,000.

		Log. Number of organisms/ml.						Ratio Strep.:Staph.	
		Pure cultures		Mixed culture					
						Total			
Exp.	Time (hours)	Strep.	Staph.	Strep.	Staph.	Direct	Calculated	Pure cultures	Mixed culture
A	0·00	3·03	3·74	3·07	3·67	3·78	3·78	1:5	1:4
	2·00	*	4·05	*	4·11	4·32			
	4·25	5·01	5·05	*	4·96	5·32		1:1	
	6·25	6·35	6·29	6·45	6·16	6·72	6·63	1:1	2:1
	8·25	7·97	7·53	8·02	7·23	8·08	8·09	3:1	6:1
	11·50	8·71	7·94	8·81	7·29	8·88	8·83	6:1	33:1
	14·25	8·68	8·21	8·85	7·27	8·86	8·86	3:1	38:1
	17·25	8·75	8·32	8·92	7·41	9·00	8·94	3:1	32:1
	25·50	8·77	8·45	8·82	7·77	8·90	8·86	2:1	11:1
	32·00	8·77	8·38	*	7·80	8·91		2:1	
	50·00	7·95	8·20	8·48	7·72	8·53	8·55	1:2	6:1
B	0·00	4·13	2·62	4·11	2·59	4·13	4·11	32:1	33:1
	2·25	5·29	3·11	5·00	2·99	5·05	5·00	150:1	100:1
	4·00	*	4·06	6·24	3·89	6·25	6·25		220:1
	6·25	8·28	5·17	8·01	4·90	8·01	8·01	1,300:1	1,300:1
	8·25	8·68	6·23	8·63	4·89	8·66	8·63	280:1	5,500:1
	11·25	8·72	7·66	8·79	5·08	8·66	8·79	11:1	5,100:1
	14·25	8·80	8·03	8·80	5·21	8·81	8·79	6:1	3,900:1
	17·00	8·79	8·24	8·88	5·30	8·89	8·88	4:1	3,800:1
	26·75	8·57	8·23	8·62	7·40	8·72	8·65	2:1	17:1
	52·00	Contaminated		7·70	7·72	8·08	8·01		1:1
C	0·00	2·45	4·26	2·48	4·29	4·28	4·30	1:64	1:64
	2·25	4·11	5·17	3·84	5·08	5·05	5·11	1:11	1:17
	4·00	5·39	6·05	5·25	5·96	6·02	6·04	1:5	1:6
	6·00	7·16	7·12	6·94	7·02	7·30	7·28	1:1	1:1
	8·25	8·53	7·72	8·50	7·42	8·54	8·53	7:1	12:1
	13·00	8·73	8·13	8·84	7·26	8·88	8·85	4:1	38:1
	16·00	8·85	8·20	8·86	7·26	8·79	8·87	5:1	40:1
	25·50	8·79	8·18	8·92	7·79	*	8·96	4:1	13:1
	50·00	7·24	7·63	7·90	7·58	8·12	8·07	1:2	2:1

TABLE 4 (continued).

Exp.	Time (hours)	Log. Number of organisms/ml.						Ratio Strep.:Staph.	
		Pure cultures		Mixed culture					
						Total			
		Strep.	Staph.	Strep.	Staph.	Direct	Calculated	Pure cultures	Mixed culture
D	0·00	6·36		6·41		6·41	6·41		
	1·50	6·90	3·92	6·88	3·93	6·84	6·88	960:1	890:1
	3·75	8·20	4·71	8·08	4·36	8·23	8·08	3,100:1	5,200:1
	6·00	—	5·84	—	—	—			
	10·00	8·69	7·60	8·68	4·81	8·70	8·68	12:1	7,400:1
	14·50	8·67	8·01	8·71	5·31	8·73	8·71	5:1	2,500:1
	21·00	—	—	8·67	*	8·67			
	26·00	8·60	7·74	8·71	7·63	8·76	8·74	7:1	12:1
	29·00	—	—	8·72	7·83	8·79	8·77		8:1
	32·00	—	—	8·69	7·97	8·75	8·77		5·1
	50·00	7·28	7·53	8·17	7·93	8·33	8·37		2:1
E	0·00		5·77		5·80	5·80	5·80		
	3·75	2·83	7·41	2·86	7·48	7·45	7·48	1:38,000	1:42,000
	6·25	3·68	7·92	4·20	7·89	7·93	7·89	1:17,000	1:5,000
	8·00	4·69	8·18	5·48	8·16	8·17	8·16	1:3,100	1:480
	10·00	5·83	—	6·74	8·12	8·13	8·14		1:24
	12·00	7·08	—	7·81	8·08	8·26	8·27		1:2
	14·25	8·57	8·05	8·48	8·05	8·64	8·62	3:1	3:1
	17·00	8·67	—	8·51	7·92	8·54	8·61		4:1
	28·00	8·79	7·81	8·49	7·73	8·59	8·56	10:1	6:1
	52·00	8·02	7·61	7·95	7·54	8·09	8·09	3:1	3:1

* Flasks unsatisfactory for obtaining counts. — No samples taken for counts.

During the experiments, inocula from low dilutions of each pure culture were sown in the selective agar to which the organism was susceptible. The only instances of growth in these flasks were those in which the inocula exceeded the safe limits as previously determined. These observations indicate that the relationship between the sensitivities of the organisms and the selective agars which were inhibitory to them was not modified by the age of the organisms. Examination of the data from the growth curves revealed that the highest number of staphylococci in mixed inocula sown in CV agar from which the streptococcal estimates were obtained was approximately 3×10^6 and that the highest number of streptococci in mixed inocula sown in SA agar from which the staphylococcal estimates were obtained was approximately 8×10^5. Both of these figures were within the safe limits as previously determined in the experiments with static populations.

Statistical Results.

Analyses of Counts as a Whole.

During the course of these investigations the results of 1,600 replicate colony counts were collected. In the early stages of the work when a number of preliminary growth curves were carried out, the counts were mainly made

in duplicate. In later work and in the growth curves presented here, triplicate counts were done. The P values from χ^2 determinations of all of the counts were examined in order to provide evidence for assessing the overall validity of the method.

TABLE 5.

Data from mixed growths in nutrient broth at 37° C. of a viridans streptococcus and Staph. albus.

Data for experiments as a whole shown in Table 4.

	Log. number organisms/ml.			
	At beginning of association		At approximate time when Staph. began to be suppressed	
Exp.	Strep.	Staph.	Strep.	Staph.
A	3·07	3·67	8·0	7·2
B	4·11	2·59	8·0	4·9
C	2·48	4·29	8·0	7·3
D	6·88	3·93	8·1	4·2
E	2·86	7·48	No suppression	

TABLE 6.

Observed and expected frequencies of probability (P) *values corresponding to* χ^2 *values of 1,600 replicate colony counts of a viridans streptococcus and* Staph. albus.

Groupings from which values of tables were derived are shown in Table 7.

		Before deletion of P values of $<\cdot01$ and $>\cdot99$		After deletion of P values of $<\cdot01$ and $>\cdot99$	
Range of P	Observed	Expected	$\frac{(\text{Obs.}-\text{Exp.})^2}{\text{Exp.}}$	Expected	$\frac{(\text{Obs.}-\text{Exp.})^2}{\text{Exp.}}$
$<\cdot01$	47	16	60·062	—	—
·01–·02	21	16	1·562	15·4	2·036
·02–·05	39	48	1·688	46·3	1·151
·05–·10	76	80	0·200	77·2	0·019
·10–·20	132	160	4·900	154·5	3·276
·20–·30	150	160	0·625	154·5	0·131
·30–·50	323	320	0·028	309·0	0·634
·50–·70	310	320	0·313	309·0	0·003
·70–·80	174	160	1·225	154·5	2·461
·80–·90	160	160	0·000	154·5	0·196
·90–·95	71	80	1·013	77·2	0·498
·95–·98	42	48	0·750	46·3	0·399
·98–·99	16	16	0·000	15·4	0·023
$>\cdot99$	39	16	33·062	—	—
Totals	1,600	1,600	105·428	1,513·8	10·827

	$\Sigma\frac{(\text{Obs.}-\text{Exp.})^2}{\text{Exp.}}$	d.f.	P. (Goodness of Fit)
Before deletion of P values of $<\cdot01$ and $>\cdot99$	105·428	13	$<\cdot001$
After deletion of P values of $<\cdot01$ and $>\cdot99$	10·827	11	·46

It may be seen from Table 6 which shows in the form of a frequency distribution, the observed and expected P values of the 1,600 counts as a whole, that the excessive frequencies of the two tail groups were responsible for the unsatisfactory Goodness of Fit and that after their removal the Goodness of Fit for the remainder of the values was highly satisfactory.

TABLE 7.

Data from frequency distributions of observed and expected probability (P) *values corresponding to* χ^2 *values of 1,600 replicate colony counts of a viridans streptococcus and* Staph. albus.

Grouped with respect to (a) organisms and media, (b) replicates and (c) means. The frequency distributions were arranged and the Goodness of Fit P values determined as for data of Table 6.
Concentration of crystal violet (CV) = 1/1,000,000. Concentration of sulphanilamide (SA) = 1/3,000.

	Grouping		Before deletion of P values of $<\cdot01$ and $>\cdot99$		After deletion of P values of $<\cdot01$ and $>\cdot99$		Observed p.c. of P values	
			Number of counts	P (Goodness of Fit)	Number of counts	P (Goodness of Fit)	$<\cdot01$ (Expected 1·0 p.c.)	$>\cdot99$ (Expected 1·0 p.c.)
	Strep.	Nutrient agar	276	·37	263	·66	1·4	3·3
		CV agar	186	·33	177	·32	2·2	2·7
		CV agar (Staph. present)*	208	·95	197	·68	3·8	1·4
(a)	Staph.	Nutrient agar	266	·58	252	·90	2·6	2·6
		SA agar	209	$<\cdot001$	190	·17	8·1	0·95
		SA agar (Strep. present)†	244	·03	227	·54	2·5	4·5
	Strep. and Staph.‡	Nutrient agar	211	·52	208	·56	0·5	0·90
(b)	Replicates	2	330	$<\cdot001$	294	·02	2·4	8·5
		3	1,100	$<\cdot001$	1,062	·27	2·5	0·91
		5	170	$<\cdot001$	158	·15	6·5	0·59
(c)	Means	1–80	800	$<\cdot001$	755	·47	2·1	3·5
		81–160	416	$<\cdot001$	394	·89	3·8	1·4
		161–320	308	·03	294	·90	3·2	1·3
		321–640	76	·22	71	·10	5·3	1·3
		Total	1,600	$<\cdot001$	1,514	·46	2·9	2·4

Number of staphylococci (*) and streptococci (†) in flasks of the counts varied from 0 to several millions (see text). ‡ Ratio of the two organisms in the counts was variable (see text).

Table 7 shows data from frequency distributions of the P values of the various groupings of counts from which the total of 1,600 were compiled. Such a procedure enabled the Goodness of Fit P values for these groupings to be examined individually and the major sources of excessively variable and excessively homogenous counts to be traced. Examination of these data showed that the major source of $P < \cdot01$ values was the staphylococcus-SA agar group and that of this group, the 5-flask counts with high means were the most heavily involved. The association of the low P values with high means must be considered fortuitous as most of the inocula for 5-flask counts were arranged to give such means. Examination of the counts as a whole which yielded $P < \cdot01$ values showed that in most cases they arose from

5-flask sets and that the extreme divergence of one of the replicate counts rather than excessive variance of the set as a whole had been the responsible factor. The divergence, moreover, was always towards zero. The relative prevalence of this one flask divergence in the staphylococcus-SA agar group however, cannot be explained. Grouping the P values with respect to means and the number of replicate counts per set showed that the excess of high P values ($P > \cdot 99$) arose entirely from duplicate counts and particularly from the lower mean groups of these counts. Of the 39 $P > \cdot 99$ values, 28 arose from duplicate counts which were identical (i.e. $\chi^2 = 0$). The prevalence of high P values among such counts would seem due to the limitation of the χ^2 formula in dealing with discontinuous variates. The expectation of identical counts in a set increases as the number of replicates decreases and also as the mean decreases. It would seem that some modification of the χ^2 formula is desirable where duplicate colony counts are being tested.

TABLE 8.

Observed and expected frequencies of probability (P) *values from* χ^2 *analyses of 130 independent sets of parallel colony counts of a viridans streptococcus in nutrient and crystal violet agars.*

Replicate inocula of the streptococcus sown in 6 flasks (3 of nutrient agar and 3 of crystal violet agar). Concentration of crystal violet = 1:1,000,000.

	Frequency					
	Observed					
Range of P	Nutrient agar	Crystal violet agar	Within treatments	Between treatments	Total	Expected*
$< \cdot 01$	2	2	1	3	3	1·3
·01–·02	2	1	2	2	1	1·3
·02–·05	2	3	5	5	2	3·9
·05–·1	9	3	1	3	7	6·5
·1 –·2	8	8	9	13	8	13·0
·2 –·3	13	11	16	14	10	13·0
·3 –·5	29	27	18	25	26	26·0
·5 –·7	27	37	36	21	30	26·0
·7 –·8	13	9	17	19	17	13·0
·8 –·9	15	18	17	16	14	13·0
·9 –·95	4	4	5	6	7	6·5
·95–·98	4	3	1	0	4	3·9
·98–·99	1	3	1	0	1	1·3
$> \cdot 99$	1	1	1	3	0	1·3
Totals	130	130	130	130	130	130
P † (Goodness of Fit)	·87	·16	·04	·33	·82	

* The frequency values of this column apply to each of the observed frequency columns.

† Tail groups from $< \cdot 01$–·05 and from ·95–$> \cdot 99$ combined for determination of Goodness of Fit P values.

In a number of cases the Goodness of Fit P values for the unadjusted groupings shown in Table 7 were satisfactory and all were satisfactory after deletion of the P values of $< \cdot 01$ and $> \cdot 99$. The percentage of counts showing excessive variation was considered to be insignificant and the analysis showed that the majority of counts of every grouping considered

varied as small samples from Poisson distributions. Such results provide satisfactory evidence that the means of the replicate counts were reliable estimates of the populations they were intended to measure.

Comparison of the Nutrient and Selective Agars.

During the growth curves, as explained previously, each pure culture was enumerated in both nutrient agar and the selective agar to which it was resistant. Treatment effects were assessed for these comparable counts of which there were for each organism, 130 sets. Tables 8 and 9 show the frequency distributions of P values from χ^2 analyses of the CV agar-nutrient agar and SA agar-nutrient agar comparisons respectively.

TABLE 9.

Observed and expected frequencies of probability (P) *values from* χ^2 *analyses of 130 independent sets of parallel colony counts of* Staph. albus *in nutrient and sulphanilamide (SA) agars.*

Replicate inocula of the staphylococcus sown in 6 flasks (3 of nutrient agar and 3 of sulphanilamide agar). Concentration of sulphanilamide = 1:3,000.

	Frequency					
	Observed					
Range of P	Nutrient agar	SA agar	Within treatments	Between treatments	Total	Expected*
$<\cdot01$	7	6	9	12	14	1·3
·01–·02	1	4	1	3	2	1·3
·02–·05	5	5	3	3	5	3·9
·05–·1	9	6	9	2	7	6·5
·1 –·2	10	10	18	18	15	13
·2 –·3	12	13	14	15	13	13
·3 –·5	26	24	25	33	19	26
·5 –·7	19	28	13	22	24	26
·7 –·8	19	12	11	6	6	13
·8 –·9	11	13	13	9	13	13
·9 –·95	4	2	8	6	8	6·5
·95–·98	4	5	5	0	2	3·9
·98–·99	2	1	1	1	1	1·3
$>\cdot99$	1	1	0	0	1	1·3
Totals	130	130	130	130	130	130
P † (Goodness of Fit)	·12	·08	·05	$<\cdot001$	$<\cdot001$	
Goodness of Fit P values after deletion of P values of $<\cdot01$ and $>\cdot99$						
Totals	122	123	121	118	115	
P ‡ (Goodness of Fit)	·53	·61	·29	·05	·57	

* The frequency values of this column apply to each of the observed frequency columns.

† Tail groups from $<\cdot01$–·05 and from ·95–$>\cdot99$ combined for determination of Goodness of Fit P values.

‡ Tail groups from ·01–·05 and from ·95–·99 combined for determination of Goodness of Fit P values.

In the case of the SA agar-nutrient agar comparison, the two Goodness of Fit P values for the frequency distributions were determined as there was an excessive number of low P values. This was not the case with the CV agar-nutrient agar comparison and only the one series of Goodness of Fit P values was determined.

(a) *Streptococcus in nutrient agar and in CV agar.* The Goodness of Fit P values shown in Table 8 indicate that the counts of the streptococcus in each medium varied as small Poisson samples and that throughout the investigation, chance differences only occurred between the two media in the recovery of the organism.

(b) *Staphylococcus in nutrient agar and in SA agar.* While the Goodness of Fit P values (Table 9) for each treatment were reasonable, the departure from the expected was due mainly to the excess of $P < \cdot 01$ values. After their removal the fits are seen to be more satisfactory. The excess of low P values in the nutrient agar treatment is a feature of this particular series rather than of the staphylococcus-nutrient agar group as a whole (see Table 7). The excess of low P values in the SA agar treatment is, generally speaking, characteristic of the group as a whole (see Table 7). The occurrence of a divergent count within either treatment contributes to the low P value intervals of the other three columns, thus explaining the higher frequencies of $P < \cdot 01$ values in these columns. After removal of the tail groups, the Goodness of Fit P values are seen to be satisfactory, showing that in the majority of cases of the recovery of the staphylococcus, chance differences only occurred between the two media.

The values of Tables 8 and 9 were derived from counts of the pure cultures during all phases of their growth. They reveal no evidence to indicate that the relationship between the sensitivities of the organisms and the selective media in which they were enumerated was modified by the age of the organisms. The results of the analyses show that the pooling of the nutrient agar and selective agar counts in the establishment of the growth curves for the pure cultures was a justifiable procedure.

DISCUSSION.

When the enumeration of the strains in mixed bacterial populations is determined only by the ability of the organisms to colonize in certain media and to be inhibited in others, strict statistical control of the method is essential.

In the experiments recorded here, statistical analysis revealed that, within certain limits, the enumeration of the streptococcus and staphylococcus in freshly prepared mixtures was quantitatively valid. The identity of the colonies counted was assumed, but the design of the experiments was such as to leave no reasonable doubt that this assumption was justified.

Evidence for the specificity and accuracy of the selective agar counts of the two organisms growing in association was, under some circumstances, direct. For example, when the ratio between the two organisms growing in association was of the order of 1:10, or less, there was an opportunity for checking the nutrient agar counts with the sum of those from the selective agars, since under these circumstances replicate inocula of the mixed culture were sown in the three media. Also at high ratios, when the nutrient agar was sown with virtually no cells of the numerically inferior organism, it was possible to test the agreement between the counts from this medium and those from the selective agar employed to enumerate the numerically superior organ-

ism. Examination of the growth curve data revealed that agreement was satisfactory in each case.

Direct confirmation of the accuracy of the counts was lacking only in the case of the numerically inferior organism and this only when the organism was significantly outnumbered by the second strain. From the results of the pure culture curves, there was no evidence that the relationship between the sensitivities of the organisms and the selective media was modified by the age of the organisms. The χ^2 values of the counts from the selective media employed to enumerate the numerically inferior organisms in the mixtures were reasonable and the curves from these estimates were smooth. These observations together with the results of the experiments with populations of known numbers would seem to leave little doubt that the estimates of the numerically inferior organisms in the mixed cultures were reliable.

The blood plate cultures (tests for contamination) indicated roughly the quantitative relationships in the mixed cultures, though when the ratio of the two organisms as revealed by the selective agars was $>1:100$ it was common to find colonies of only the numerically superior organism on the plate. This observation is consistent with the experience of Fulton (1937) who was unable to record ratios of higher than 1:100 when using colonial appearance in surface platings to follow the population changes in mixed cultures of *Escherichia coli* and *Salmonella schottmuelleri*.

It is worth recording that although colonial characteristics were not used as a basis for enumerating the two strains in this work, there was a distinct difference in appearance, both in nutrient and the selective agars, between the streptococcal and staphylococcal colonies.

It is usually considered that replicate colony counts made from bacteria which form clumps in suspension, do not conform to Poisson samples. In this work no attempt was made to study the "clump sizes" or "chain lengths" of the organisms concerned. Each organism gave a macroscopically smooth and stable turbidity in the particular medium used. In stained smears the streptococcus occurred mainly in pairs and occasionally in chains of three, four, or five cells, while the staphylococcus occurred mainly as single cells with occasional pairs and small clumps. Statistical analysis showed that the colony counts of each organism varied as small Poisson samples, indicating that the reproductive units, whatever their nature, were distributed in a Poisson series.

The statistical evidence from the counts considered as a whole provides a sound basis for the use of flasks as a satisfactory substitute for Petri plates.

At present no explanations can be advanced for the phenomena observed during the associations of the streptococcus and the staphylococcus. The suppression of the staphylococcus in the associations appeared to be correlated with the heavy concentration of streptococcal cells occurring towards the end of the logarithmic phase and during the early part of the stationary phase and

was possibly due to the liberation of toxic products. The stimulation of the streptococcal growth in the presence of a heavy concentration of the staphylococcus was possibly due to some growth factor supplied by the staphylococcus. In earlier experiments, not reported here, which were conducted in a less nutritive broth, this stimulation was intense.

SUMMARY.

Differences in sensitivity of *Staphylococcus albus* and a viridans streptococcus to crystal violet and sulphanilamide in nutrient agar have enabled enumeration of the viable organisms of the two strains to be made both on freshly prepared mixtures and during association in broth culture.

In crystal violet agar, to which the streptococcus was more resistant than the staphylococcus, the streptococcus could be enumerated in the presence of approximately 20,000,000 staphylococci. In sulphanilamide agar to which the staphylococcus was more resistant than the streptococcus, the staphylococcus could be enumerated in the presence of approximately 1,000,000 streptococci.

Some features associated with the mixed growths of the organisms are discussed.

Statistical control of the method was achieved by procedures which recognized the ideal bacterial colony count as a Poisson variate.

Acknowledgments. This research work was carried out at the instigation of the late Dr. A. E. Platt, while Director of the Institute of Epidemiology and Preventive Medicine. The authors are much indebted to him for his criticism and advice. Grateful thanks are also expressed to Dr. H. O. Lancaster for guidance in the statistical methods, and to Mr. N. F. Stanley, Mr. W. J. Scott and Miss Joan Norris for helpful suggestions and criticism.

REFERENCES.

Fisher, R. A., Thornton, B. A. and Mackenzie, W. A. (1922): Ann. appl. Biol., 9, p. 325.
Fulton, M. (1937): J. Bact., 34, p. 301.
Pike, R. M. and Foster, A. Z. (1944): *Ibid.*, 47, p. 97.
Woods, D. D. (1947): Ann. Rev. Biochem., 16, p. 605.

THE PREPARATION AND PROPERTIES OF SOMATIC ANTIGENS ISOLATED FROM *BACTERIUM COLI*

By LILA HAYES AND NEVILLE F. STANLEY

(From the Institute of Epidemiology and Preventive Medicine, Prince Henry Hospital, Sydney).

(Accepted for publication 11th October, 1949.)

Since the important contributions to the study of bacterial endotoxins were made by the French and English workers (Boivin and Mesrobeanu, 1933, 1934; Raistrick and Topley, 1934; Delafield, 1934) with *Salmonella typhi-murium*, the somatic antigens from Gram-negative bacilli have received the attention of numerous investigators. Although the methods of isolation of the toxic antigenic complexes have varied considerably the general biological reactions of the compounds have been much the same from such organisms as *Salmonella typhi* (H. R. Morgan, 1940, 1941, 1943, 1948a, 1948b; Favorite and H. R. Morgan, 1943), *Shigella dysenteriae* (W. T. J. Morgan and Partridge, 1940, 1941; Tal and Olitzki, 1948), *Shigella paradysenteriae* (Flexner), (Goebel *et al.*, 1945; Smolens *et al.*, 1946) and *Shigella sonnei* (Baker *et al.*, 1949). In comparison *Bact. coli* has received little investigation (Boivin *et al.*, 1942, 1946).

The following study describes preliminary investigations undertaken in an attempt to compare the biological properties of certain bacterial fractions, and in particular, polysaccharide complexes. Although strains of *Bact. coli* antigenically related to organisms of the genus *Salmonella* or *Shigella* have been recorded (Edwards *et al.*, 1942; Boivin *et al.*, 1942; Seligmann and Saphra, 1946), the strain of *Bact. coli* used in these investigations possessed no antigenic relationship as shown by cross agglutination reactions to salmonella types commonly isolated in this laboratory (*Salmonella typhi-murium* and *Salmonella bovis-morbificans*). The somatic antigens isolated from this strain of *Bact. coli* are shown to be impure polysaccharides. Experiments with rabbits indicate that the fractions have certain biological properties in common with polysaccharide complexes isolated by previous workers from organisms of the genera *Salmonella* and *Shigella*.

MATERIALS AND METHODS.

Rabbits: White rabbits weighing from 2–2·5 kg. were used in all rabbit experiments. Blood samples for total and differential leucocyte and erythrocyte counts, haemoglobin, blood sugar and blood lactic acid estimations were collected from the marginal ear vein.

Chemical tests: Osborne's modification[1] of the biuret test was used for the detection of small amounts of protein. Stumpf's (1947) modification of the Dische test was used for the detection of desoxyribonucleic acid and Mejbaum's (1939) modification of Bial's test for the presence of pentose. The presence of phosphorus was determined by the method of Simonsen *et al.*, (1946). Blood sugars were estimated by the method of Hagedorn and Jensen and blood lactic acid values by the micro-method of Hawk *et al.*, (1947). The method of Somogyi was used for the estimations of reducing sugars formed on acid hydrolysis of the polysaccharides.

EXPERIMENTAL.

Isolation of the Polysaccharide Fractions.

The polysaccharides were prepared by two methods which differed mainly in the manner of disintegration of the bacterial cells. In method 1 the cells were disrupted mechanically by grinding with sand while in method 2 the cells were lysed with glycine after the method of Maculla and Cowles (1948). The properties of the polysaccharide prepared by method 1 (fraction M) are described fully in this paper. Although the polysaccharide prepared by method 2 (fraction G) has not been studied as extensively as fraction M, the investigations that have been made indicate that the two fractions are not identical.

(a) Method 1.

The strain of *Bact. coli* was grown on tryptic digest agar in trays which were inoculated from a 24-hour broth culture. After incubation of the agar cultures at 37° C. for 18 hours, the growth was washed off in distilled water. The cells were removed by centrifugation, washed in distilled water, recovered by further centrifugation and killed by heat at 55° C. for 30 minutes. The packed bacterial cells were extracted three times with an ethanol-ether mixture (equal parts), then dried and ground with sand. The disrupted bacterial cells were extracted three times with distilled water, the aqueous extracts being recovered by centrifugation. The deposit was discarded. Two volumes of ethanol containing a trace of sodium acetate were added to the supernatant and the resulting precipitate extracted three times with distilled water. Material precipitated by two volumes of ethanol was again obtained, dissolved in distilled water and centrifuged.

The deposit was discarded. The aqueous solution was extracted with chloroform-amyl alcohol mixture (Sevag *et al.*, 1938) seven times after which no gel was formed. The aqueous solution was precipitated twice more with ethanol-sodium acetate. The final precipitate was washed three times with alcohol, three times with ether and dried. It appeared as a fine white powder (fraction M).

(b) Method 2.

The strain of *Bact. coli* was grown on tryptic digest agar in trays which were inoculated from a 24-hour broth culture. After incubation at 37° C. for 6 hours, the growth was washed off with saline at 37° C. Sufficient of a 3 M glycine solution at pH 8 and at 37° C. was then added to the thick bacterial suspension to give a final glycine concentration of 1 M. The mixture was well-shaken and incubated in a water bath at 40° C. for 24 hours. The suspension, which had cleared considerably, was then dialyzed against glass-distilled water to remove the glycine. Two changes of water were made over a period of 48 hours. The material was centrifuged and the deposit discarded. Two volumes of ethanol containing a trace of sodium acetate were added to the supernatant and the resulting precipitate extracted twice with dis-

[1] Official and tentative Methods of Analysis of the Association of Official Agricultural Chemists (1940), p. 354.

tilled water. Material precipitated by two volumes of ethanol was again obtained, dissolved in distilled water and centrifuged. The deposit was discarded. The aqueous solution was extracted eight times with chloroform-amyl alcohol mixture and then treated as in Method 1. The final product was a pale brown lumpy material (fraction G).

Chemistry.

Both fraction M and fraction G were readily soluble in warm water. Chemical tests (Table 1) revealed certain differences between the two fractions.

TABLE 1.

Chemical examination of fractions M and G.

Fraction	Molisch	Biuret	Osborne's biuret	Sakaguchi	Millon	Dische	Bial	Nitrogen	Phosphorus	Sulphur	p.c. Reducing sugars
M	++	N	±	+	++	+	±	+	+	±	13·5
G	++	N	tr.	tr.	N	N	++	++	+	tr.	38·4

++ Strong positive. + Positive. ± Weak positive. tr. Trace. N Negative.

The chemical facts presented show clearly the presence of a carbohydrate nucleus in both fractions. Intact protein is absent, but nitrogen compounds, probably of a peptide nature are definitely present. Bial's test for pentose is more marked with fraction G than fraction M and the percentage of reducing sugars formed on hydrolysis is higher. The absence of phenolic substances (Millon's reaction) and desoxyribonucleic acid (Dische test) from fraction G indicates further chemical differences between the two fractions.

Preparation of Immune Sera.

One rabbit received 7 doses of heat-killed vaccine over a period of 11 days; the initial dose of 0·1 ml. of a suspension containing 500×10^6 organisms per ml. was increased to the final dose of 1·0 ml. of $1{,}000 \times 10^6$ organisms per ml.

A second rabbit received a total of 1·60 mg. of polysaccharide (fraction M) in 8 doses over a period of 16 days; the initial dose of 0·05 mg. which produced no severe reactions, was gradually increased to the final dose of 0·5 mg.

Both animals were rested for one week, bled, and the serum, with the addition of merthiolate, was stored in the refrigerator. The two rabbits were reserved for further experiments.

Toxicity of the Polysaccharides for Rabbits.

Intravenous injection of 0·2–0·25 mg. of fraction M into 3 rabbits proved lethal in each case. Within 2–3 hours the animals showed prostration and diarrhoea; the peripheral circulation was depressed and within 3–18 hours the animals were dead. Prior to death heart blood was collected; examination of this showed a state of marked hyperglycaemia, high blood lactic acid level and leucopenia. Injection of a smaller dose, 0·1 mg., which was not lethal, produced the same toxic symptoms in 2–3 hours, with changes in the cells of the venous blood, blood sugar and blood lactic acid levels (see below). None of the rabbits showed any significant changes in temperature following injection of the polysaccharide. Rabbits failed to respond in any of the above ways to doses of polysaccharide, as high as 12 mg. given by mouth.

Fraction G proved more toxic for rabbits than fraction M; intravenous injection of 0·05 mg. produced death within three hours with symptoms similar to those produced by fraction M.

TABLE 2.

Response of normal rabbits to intravenous injection with Bact. coli *polysaccharide (fraction M); 0·05 mg. per kg. of body weight.*

Rabbit	Time of counts	Total Leucocytes*	Lymphocytes*		Polymorphonuclear cells*		Monocytes*		Erythrocytes*	Hb. gm. per 100 ml.	Blood sugar†	Blood lactic acid†	Rectal Temp. °F
			p.c.	absolute	p.c.	absolute	p.c.	absolute					
I	before injection	12,200	53	6,500	43	5,200	4	500	$5·4 \times 10^6$	12·1	101	—	97·6
	after 2½ hours	2,300	55	1,300	45	1,000	0	0	6·0	12·1	170	—	95·4
	after 24 hours	50,000	7	3,500	90	45,000	3	1,500	5·2	9·3	74	—	98·6
	after 47 hours	49,700	13	6,500	85	42,200	2	1,000	5·2	9·3	118	—	96·5
II	before injection	10,000	70	7,000	25	2,500	5	500	—	—	118	24·4	96·6
	after 2½ hours	3,300	80	2,600	20	700	0	0	—	—	184	125	95·0
	after 6 hours	9,300	40	3,700	58	5,400	2	200	—	—	146	50	95·0
	after 24 hours	32,000	27	8,600	73	23,400	0	0	—	—	—	—	95·4
	after 49 hours	44,200	10	4,400	86	38,000	4	1,800	—	—	60	48	95·2
	after 72 hours	animal dead											

* per cubic millimetre blood. — test not performed. † milligrams per 100 millilitres blood.
Hb. = haemoglobin.

Changes in the Cells of Venous Blood of Normal and Immune Rabbits Following Intravenous Injection of the Polysaccharides.

On injection of 0·05 mg. of fraction M per kg. body weight (approximately 0·1 mg.) normal rabbits became obviously ill within 2 hours and exhibited a leucopenia characterized by loss of polmorphonuclear cells. Twenty-four hours after injection there was a marked neutrophil leucocytosis (Table 2 and Fig. 1). Erythrocyte and haemoglobin levels remained normal and there were no significant temperature changes.

Intravenous injection of 0·005 mg. of fraction G into a rabbit produced the characteristic leucopenia followed by leucocytosis; no change in erythrocytes or haemoglobin occurred but the animal's temperature rose from 98° F. (pre-injection level) to 102·2° F. at 4 hours.

Intravenous injection of 0·05 mg. of fraction M into a normal rabbit produced 12 hours after injection a slight leucocytosis which became more marked at 24 hours. Repeated injection of small but increasing doses of polysaccharide at intervals of not more than 3 days, as in the immunizing process produced a neutrophil leucocytosis which was maintained for the period of the injections. Towards the end of the immunizing period, when the rabbit could tolerate up to 0·5 mg. polysaccharide, the total leucocyte count began to drop back to normal. Seven days after the last immunizing dose the rabbit showed a normal blood picture.

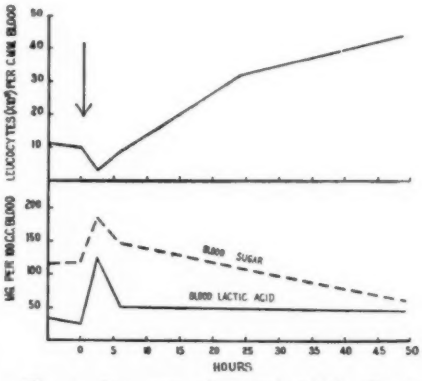

Fig. 1. Response of normal rabbit (II) to injection with polysaccharide (fraction M).

The degree of tolerance exhibited by the immune rabbit to further injection of fraction M depended on the amount of polysaccharide given and the time since the last injection. At the completion of immunization, the rabbit was rested for 4 weeks and then given a further 0·5 mg. dose of polysaccharide. Within 2 hours the rabbit exhibited a leucopenia characterized by loss of polymorphonuclear cells; the peripheral circulation was depressed, but there was no diarrhoea. Twenty-four hours after the injection there was a neutrophil leucocytosis. A further 0·5 mg. of polysaccharide given 24 hours after the first, produced similar results. The rabbit received further doses on the fourth and sixth days; the blood picture on these days indicated that the animal was again developing a tolerance to the polysaccharide. By the seventh day the blood picture was normal (Table 3).

The rabbit was then rested for 11 weeks, after which it received 0·1 mg. of polysaccharide. Within 2 hours the animal showed the characteristic leucopenia; the peripheral circulation was depressed but there was no diarrhoea. Twenty-four hours later there was a marked neutrophil leucocytosis. There were no significant changes in erythrocyte and haemoglobin levels.

The rabbit immunized with *Bact. coli* vaccine received 0·05 mg. fraction M per kg. body weight, three weeks after the last immunizing injection. Within 2 hours the animal showed a leucopenia characterized mainly by loss of lymphocytes; the peripheral circulation was depressed but there was no diarrhoea. Within 24 hours there was a marked neutrophil leucocytosis. No significant changes were recorded in erythrocyte and haemoglobin levels (Table 4). A further 0·05 mg. dose of polysaccharide 8 days later produced similar results.

TABLE 3.

Rabbit immunized with Bact. coli *polysaccharide (fraction M)—response to further injections of polysaccharide.*

Dose	Day	Time of counts	Total leucocytes*	Lymphocytes*		Polymorphonuclear cells*		Monocytes*	
				p.c.	absolute	p.c.	absolute	p.c.	absolute*
0·5 mg.	1st	before injection	8,000	56	4,500	40	3,200	4	300
		after 2 hours	3,000	84	2,500	16	500	0	0
0·5 mg.	2nd	before injection	22,700	7	1,600	88	20,000	5	1,100
		after 2 hours	2,800	53	1,500	42	1,200	5	100
—	3rd	—	22,200	15	3,300	84	18,700	1	200
0·5 mg.	4th	before injection	14,200	46	6,500	52	7,400	2	300
		after 2 hours	9,100	37	3,400	62	5,600	1	100
—	5th	—	11,400	48	5,500	51	5,800	1	100
0·5 mg.	6th	before injection	7,600	54	4,100	42	3,200	4	300
		after 2 hours	6,500	46	3,000	53	3,400	1	100
—	7th	—	8,600	50	4,300	48	4,100	2	200

* per cubic millimetre.

TABLE 4.

Rabbit immunized with Bact. coli *vaccine—response to intravenous injection with polysaccharide (fraction M); 0·05 mg. per kg. body weight.*

Time of counts	Total leucocytes*	Lymphocytes*		Polymorphonuclear cells*		Monocytes*		Erythrocytes*	Hb. gm. per 100 ml.	Blood sugar†	Rectal Temp. °F.
		p.c.	absolute	p.c.	absolute	p.c.	absolute				
before injection	7,200	56	4,000	43	3,100	1	100	$4·9 \times 10^6$	10·2	102	100·8
after 2½ hours	3,300	20	700	80	2,600	0	0	—	12·1	130	102·5
after 24 hours	30,600	9	2,800	91	27,800	0	0	4·1	8·6	98	102·6
after 48 hours	18,800	30	5,600	69	13,000	1	200	4·6	7·8	120	101·8

* per cubic millimetre of blood. † milligrams per 100 millilitres blood. — test not performed. Hb. = haemoglobin.

Changes in Blood Sugar and Blood Lactic Acid Levels of Normal and Immune Rabbits Following Intravenous Injection of the Polysaccharides.

Intravenous injection of fraction M (0·05 mg. per kg. body weight) into a normal rabbit produced a marked hyperglycaemia within 2½ hours, followed by a hypoglycaemia at 24 hours, after which the blood sugar level returned to normal (Table 2). Lactic acid estimations were omitted. A second rabbit showed similar blood sugar changes; at the time of hyperglycaemia the lactic acid level rose sharply and then fell at 6 hours to approximately twice the pre-injection level. Forty-eight hours after injection, when the rabbit showed a hypoglycaemia the lactic acid level remained elevated. The animal died within 72 hours.

Intravenous injection of 0·005 mg. of fraction G into a rabbit (dose sufficient to induce a temperature rise and leucopenia) did not produce any significant changes in blood sugar or blood lactic acid levels. Because of the marked toxicity of fraction G, a dose greater than 0·005 mg. could not be given if the animal were to survive. It is possible that the blood sugar and blood lactic acid responses are related quantitatively to the amount of polysaccharide

injected. If this be the case, the lack of such responses on injection of 0·005 mg. of fraction G, is not unexpected.

When the rabbit immunized with fraction M received 0·5 mg. of the same fraction no changes in blood sugar or lactic acid levels occurred. The rabbit immunized with *Bact. coli* vaccine showed slight changes in blood sugar levels when injected with 0·1 mg. of fraction M (Table 4). The response was insignificant compared with that given by the normal rabbit.

Lysis of Leucocytes in vitro *by Fraction M.*

A modification of the technique described by Dennis *et al.*, (1939) was used to determine the ability of the polysaccharide to lyse rabbit leucocytes *in vitro*. A rabbit known to have a neutrophil leucocytosis was selected. Five ml. of blood were collected from the marginal ear vein in a wide-mouthed test tube lined with paraffin and containing 5 mg. of heparin dissolved in a few drops of distilled water; 0·5 ml. of blood was added to duplicate small paraffin-lined tubes containing 0·5 ml. of the polysaccharide to be tested and to tubes containing 0·5 ml. saline (control). The tubes were sealed with paraffin-coated corks and incubated in a water bath at 38° C. The tubes were shaken gently every 5 minutes. Results of total and differential leucocyte counts, made at 0 and 1 hours are shown in Table 5 (averages of duplicate counts). Within 1 hour the polysaccharide at a final dilution of 1:10,000 had lysed approximately 40 p.c. of the leucocytes originally present. As in the *in vivo* experiments, the decrease in leucocytes was characterized by loss of polymorphonuclear cells.

TABLE 5.

Lysis of leucocytes in vitro *by* Bact. coli *polysaccharide* (*fraction M*).

Concentration of Polysaccharide	Nil (Control)				1:10,000				1:100,000			
Time	Total* leucocytes	L*	P*	M*	Total* leucocytes	L*	P*	M*	Total* leucocytes	L*	P*	M*
0 hours	28,000	7,000	20,000	1,000				as for	control			
1 hour	29,000	7,000	21,500	500	12,000	7,500	4,500	0	11,000	7,000	4,000	0

* all counts corrected to nearest 500 per cubic millimetre. L lymphocytes. P polymorphonuclear cells. M monocytes.

Serology.

The polysaccharide (fraction M) was antigenic. Rabbit antisera prepared against the vaccine and the polysaccharide both agglutinated a suspension of this strain of *Bact. coli* at a final dilution of 1:5,120.

Precipitation. Fraction M reacted specifically with the homologous immune sera prepared either against the whole cell or the polysaccharide. With a serum dilution of 1:40 the final dilution of the polysaccharide giving precipitation was 1:5,120,000 for both the polysaccharide and vaccine immune sera. Using a polysaccharide dilution of 1:80,000 the vaccine and polysaccharide immune sera gave precipitation at final dilutions of 1:10,240 and 1:5,120 respectively.

The precipitate in the optimal tube in the constant antigen titration was injected intravenously into rabbits. The rabbit exhibited the characteristic leucopenia and leucocytosis but no changes in temperature, erythrocytes, haemoglobin, blood sugar and blood lactic acid levels occurred.

Using a serum dilution of 1:40 the final dilution of fraction G giving precipitation was 1:20,480,000 for both the vaccine and polysaccharide antisera (prepared against fraction M). Using a fraction G dilution of 1:80,000 the vaccine and polysaccharide antisera gave precipitation at final dilutions of 1:5,120 and 1:2,560 respectively (see Table 6).

Haemagglutination reaction. A modification of the technique described by Warburton *et al.*, (1949) was used to determine the power of vaccine and polysaccharide immune sera to agglutinate sheep erythrocytes sensitized with polysaccharides.

A 3 p.c. suspension of washed sheep erythrocytes containing polysaccharide 1:10,000 was incubated at 37°C. for 2 hours. The erythrocytes were then washed three times with saline and resuspended in saline to form a 1 p.c. suspension. Serial twofold dilutions of the immune sera in 1 ml. volumes were prepared along with the necessary controls. To each tube was added 1 ml. of a 1 p.c. suspension of sensitized erythrocytes. The tubes were mixed and left at room temperature for two hours and then at 4° C. for approximately 16 hours or overnight. Final readings were made at approximately 18 hours.

The erythrocytes sensitized with fraction M were agglutinated by the vaccine and polysaccharide immune sera at final dilutions of 1:10,240 and 1:1,280 respectively.

The erythrocytes sensitized with fraction G were agglutinated by the vaccine and polysaccharide immune sera (prepared against fraction M) at final dilutions of 1:20,480 and 1:2,560 respectively (see Table 6).

TABLE 6.

Summary of serological reactions.

Fraction	Titre of antigen constant antibody 1:40		Titre of antisera constant antigen 1:80,000		Haemagglutinating titre of antisera	
	Vaccine*	Polysaccharide*	Vaccine*	Polysaccharide*	Vaccine*	Polysaccharide*
M	5,120,000	5,120,000	10,240	5,120	10,240	1,280
G	20,480,000	20,480,000	5,120	2,560	20,480	2,560

* Vaccine and Polysaccharide (fraction M) antisera.

Shwartzman phenomenon. Fraction M failed to induce the Shwartzman phenomenon in either rabbits or guinea-pigs.

DISCUSSION.

In an attempt to isolate fractions with as little degradation of the polysaccharide as was possible, methods were selected which were assumed to involve only mild chemical treatment of the antigenic complex.

The use of glycine (Maculla and Cowles, 1948) for the disruption of bacterial cells, was successfully applied to the disintegration of the *Bact. coli* cells used in the investigations described here. It proved to be more efficient in disrupting the cell than did grinding by several mechanical means (e.g. pestle and mortar, glass cone and socket joint and the ball-mill described by Langner and Forrester, 1939). Sufficient glycine could be removed by dialysis to prevent interference with the isolation of polysaccharide by alcohol precipitation.

The polysaccharide obtained from glycine-lysed cells differed in certain

chemical (see Table 1) and biological properties from the fraction prepared from *Bact. coli* cells disrupted mechanically. Apart from the glycine treatment and dialysis the only difference in the two methods was the age of the cultures used for extraction.

Unlike fraction G, fraction M produces no temperature rise following intravenous injection. Fraction M induces hyperglycaemia and a rise in blood lactic acid but fraction G does not. Both fractions induce a leucopenia followed by leucocytosis. H. R. Morgan (1940, 1941) and Favorite and H. R. Morgan (1942) have described the preparation and properties of a polysaccharide isolated from *S. typhi* by a method similar to that used for the isolation of fraction M. Intravenous injection of their fraction into rabbits induced a leucopenia followed by leucocytosis, hyperglycaemia followed by hypoglycaemia, and pyrexia. Apart from its ability to produce pyrexia this fraction has biological properties similar to fraction M. Delafield (1934) has described similar blood sugar changes in rabbits following injection with a polysaccharide prepared from *S. typhi-murium* (Raistrick and Topley, 1934). Delafield (1934) has also described the development of a tolerance to the blood sugar response in immune rabbits on further injection of the polysaccharide. In the experiments described here a similar tolerance to the blood sugar response was observed when the rabbit immunized with fraction M received a further dose of the polysaccharide.

The development of tolerance to leucopenia by repeated doses of a polysaccharide prepared from *Sh. dysenteriae* has been described (Olitzki *et al.*, 1941). H. R. Morgan (1941) observed that rabbits immunized with a polysaccharide prepared from *S. typhi*, did not exhibit this tolerance when injected with a further dose of the fraction; he gave no indication, however, of the lapse of time between the final immunizing dose and the assaulting dose. Leucopenic tolerance was established by repeated injection of rabbits with fraction M, but the tolerance developed was not lasting (Table 3). When the tolerance no longer existed, the serum of the immune rabbit contained antibodies capable of reacting with fraction M (see Table 6). This phenomenon was also observed in rabbits immunized with *S. typhi* polysaccharide (H. R. Morgan, 1941). Morgan described the production of leucopenia in normal rabbits following injection with the precipitate formed by mixing *S. typhi* polysaccharide with antiserum. The precipitate formed by mixing fraction M with antiserum, produced, on intravenous injection, similar changes in the leucocyte count of normal rabbits, but no change in temperature, blood sugar or blood lactic acid levels. It would seem that the component of the biologically active molecule producing leucopenia is not neutralized by antiserum but the component responsible for the blood sugar and blood lactic acid responses is inactivated in the presence of antiserum. The response of the normal rabbit to the polysaccharide-antiserum precipitate is similar to that of

the immune rabbit to further injection with the polysaccharide. A factor not taken into consideration was the likelihood that the antigen-antibody complex formed *in vitro* could dissociate in the blood stream.

The fact that fraction G reacts serologically with antiserum prepared against fraction M, and with the vaccine antiserum, indicates that the two fractions have a hapten grouping, at least, in common. There is a marked difference in the haemagglutinating power of the vaccine and polysaccharide (fraction M) antisera for sheep erythrocytes, sensitized with fractions M and G (see Table 6). The higher haemagglutinating titre obtained with vaccine antiserum has also been observed with other bacterial polysaccharides at present under investigation (unpublished observations).

The differences in chemical composition between fractions M and G possibly explain the different blood sugar, blood lactic acid and temperature responses induced in rabbits on intravenous injection of the fractions. Tal and Olitzki (1948), describing antigens prepared from *Sh. dysenteriae*, stated that the leucopenia is produced by conjugated protein and undegraded polysaccharide fractions, both substances having a phosphorus group attached. Removal of the phosphorus group inactivates both the protein and the polysaccharide. Chemical tests reveal the presence of phosphorus in both fractions M and G; phosphorus attached to the carbohydrate nucleus in some manner, may possibly be responsible for the leucopenia produced in rabbits by fractions M and G. Raistrick and Topley (1934), who prepared a polysaccharide fraction from *S. typhi-murium*, suggest the importance of the presence of phosphorus and sulphur in the biologically active molecule.

SUMMARY.

The isolation and properties of two somatic antigens (denoted M and G) from *Bact. coli* are described. Fraction M was prepared from cells disrupted by grinding and fraction G from cells lysed with glycine. Chemical tests revealed that the two fractions, although mainly polysaccharide in nature, were not identical. Biological differences between the two fractions were observed and thought to be caused by (1) the use of glycine instead of mechanical means for disintegrating the bacterial cell, and (2) the use of young cells in the glycine method as opposed to older cells in the mechanical method.

The differences in behaviour of normal and immune rabbits following intravenous injection of the polysaccharides are described as well as the tolerance exhibited by animals to certain properties of the antigens. Such properties are compared with those of somatic antigens isolated by previous investigators from organisms of the genera *Salmonella* and *Shigella*.

Acknowledgment. The authors wish to express their gratitude to Mr. A. Jennings for chemical examination of the fractions.

REFERENCES.

Baker, E. E., Goebel, W. F. and Perlman, E. (1949): J. exp. Med., 89, p. 325.
Boivin, A. (1946): Ann. inst. Pasteur, 72, p. 143.
Boivin, A., Corre, L. and Lehoult, Y. (1942): Rev. d'immunol., 7, p. 97.
Boivin, A. and Mesrobeanu, L. (1933): Compt. rend. Soc. de biol., 112, p. 76.
Boivin, A. and Mesrobeanu, L. (1934): *Ibid.*, 115, p. 304.
Delafield, M. E. (1934): Brit. J. exp. Path., 15, p. 130.
Dennis, W. E. and Senekjian, H. (1939): Amer. J. Hygiene, 30 (Sect. B), p. 21.
Edwards, P. R., Cherry, W. B. and Bruner, D. W. (1943): J. infect. Dis., 73, p. 229.
Favorite, G. O. and Morgan, H. R. (1942): J. clin. Invest., 21, p. 589.
Goebel, W. F., Binkley, F. and Perlman, E. (1945): J. exp. Med., 81, pp. 315, 331, 349.
Hawk, Oser and Summerson (1947) : Practical Physiological Chemistry, p. 569.
Langner, P. H. and Forrester, J. S. (1939): J. Immunol., 37, p. 133.
Maculla, E. S. and Cowles, P. (1948): Science, 107, p. 376.
Mejbaum, W. (1939): Z. physiol. Chem., 258, p. 117.
Morgan, H. R. (1940): Proc. Soc. exp. Biol. Med., 43, p. 529.
Morgan, H. R. (1941): J. Immunol., 41, p. 161.
Morgan, H. R. (1943): Amer. J. Path., 19, p. 135.
Morgan, H. R. (1948 a): J. clin. Invest., 27, p. 706.
Morgan, H. R. (1948 b) J. Immunol., 59, p. 129.
Morgan, W. T. J. and Partridge, S. M. (1940): Biochem. J., 34, p. 169.
Morgan, W. T. J. and Partridge, S. M. (1941): *Ibid.*, 35, p. 1,140.
Olitzki, L., Avinery, S. R. and Bendersky, J. (1941): J. Immunol., 41, p. 361.
Raistrick, H. and Topley, W. W. C. (1934): Brit. J. exp. Path., 15, p. 113.
Seligmann, E. and Saphra, I. (1946): J. Immunol., 54, p. 275.
Sevag, M. G., Lackman, D. B. and Smolens, J. (1938): J. biol. Chem., 124, p. 425.
Simonsen, D. G., Westman, M., Westover, L. M. and Mahl, J. (1946): *Ibid.*, 166, p. 747.
Smolens, J., Halbert, S. P., Mudd, S., Doak, B. W. and Gonzalez, L. M. (1946): J. Immunol., 52, p. 41.
Tal, C. and Olitzki, L. (1948): *Ibid.*, 58, p. 337.
Warburton, M. F., Keogh, E. V. and Williams, S. W. (1949): Med. J. Austral., 1, p. 135.

CYTOCHEMICAL CHANGES IN EARLY ECTROMELIA INFECTION OF MICE

By P. M. De BURGH[1]

(From the Department of Bacteriology, University of Sydney).

(Accepted for publication 10th November, 1949.)

Although knowledge of the biochemical aspects of host parasite relationship of the bacterial virus is increasing, there is little connected information about such problems in mammalian virus infections. Virus infections of bacteria are more easily controlled experimentally as uniform suspensions of both host and parasite are readily available, and information about normal bacterial metabolism is accumulating rapidly. Although many aspects of mammalian cell metabolism can be investigated, the study of changes in virus-infected cells is more difficult than in infected bacterial cells. If there existed a virus which was readily isolated and measured and which attacked all the cells of some easily isolated organ, without side effects such as infiltration of inflammatory cells, then it should be possible to compare the biochemical lesions of bacterial and mammalian virus infections.

The Moscow strain of ectromelia virus fulfils some of these requirements. It multiplies rapidly in the liver (Fenner, 1949) causing no inflammatory reaction until necrosis of liver cells is produced. If large doses of virus are used, numerous necrotic foci are produced in the liver; but with very large doses of virus the changes are diffuse, frank necrosis is only recognizable just before the death of the mouse, and the majority of cells appear to be invaded by the virus. The Hampstead egg-adapted strain of ectromelia virus very rarely produces death of the mice in the period of maximum multiplication in the liver. This strain of virus multiplies less rapidly and reaches a lower titre than the Moscow strain (Fenner, 1949). This communication deals with the changes found by histochemical methods in the livers of mice infected with the Moscow and Hampstead strain of ectromelia virus.

Materials and Methods.

The strains of ectromelia virus were kindly supplied by Professor F. Fenner. Albino mice, usually males 6–10 weeks old, were used. Stock virus suspension of the Moscow strain was made from infected mouse liver and contained about 10^7 infectious particles per ml. It was distributed in tubes and stored at —15° C. For use, a tube was thawed and injected immediately after thawing, and the remainder discarded. The mice were injected in the pad of one

[1] This work was carried out under a grant from the National Health and Medical Research Council, Canberra.

or both hind feet with 0·05 ml. of virus suspension, the feet becoming swollen in 3–4 days and the mice dying in 5–8 days in the majority of cases.

The Hampstead strain was prepared similarly. The suspension contained about 10^6 infectious particles per ml. Swelling of the feet occurred usually 5–6 days after injection and the livers were examined 6–9 days after infection.

The mice were killed by cervical dislocation or decapitation and the livers rapidly removed. Pieces were fixed in cold acetone and sections made and stained for acid and alkaline phosphatase after the method of Gomori (1939). The remainder of the liver was used for other determinations.

Serial sections were cut from the acetone fixed blocks and stained in various sequences with Ehrlich's acid haematoxylin with or without eosin, for glycogen using the periodic acid-Feulgen method following the technique of McManus (1948), as well as the phosphatase methods. Inorganic phosphate was shown by treating the deparaffined section with the cobalt nitrate and ammonium sulphide solutions of the Gomori method. To demonstrate ability to form glycogen, the mice were starved overnight, then given 0·25 ml. of 20 p.c. glucose by intraperitoneal injection. After 1 hour, another injection of glucose was given and the animals killed one hour later.

RESULTS.

Following infection with the Moscow strain the mice appear well for 4–6 days. The great majority die within 24 hours of the onset of symptoms, the earlier onset being seen in those mice which were injected in both feet.

The liver shows no definite macroscopic changes until the 5th day. The main change seen then is a diffuse enlargement and the liver is paler and firmer than normal, this change being most marked in moribund animals.

The earliest histological changes are found on the 4th or 5th days. After the mice are starved overnight and given glucose by intraperitoneal injection, a section stained for glycogen shows small circular areas devoid of glycogen. These areas show no constant anatomical distribution. Adjacent sections stained by the other methods show no abnormality. In more advanced infections, these glycogen-deficient areas were larger in diameter and showed in the centre of the lesion a few cells which stained pink with the glycogen stain, as opposed to the purplish red of glycogen, (Figs. 1A and 2A). This pink staining material presumably contains carbohydrate but is not affected by salivary amylase. It has not been investigated further. Haematoxylin and eosin sections show that in these pink-staining areas, the nuclei are usually distorted or absent, sometimes intact, and the cytoplasm may stain a different shade from normal. The alkaline phosphatase activity of cells in the pink-staining areas, and sometimes in nearby apparently normal looking cells, shows a marked change. Enzyme activity appears in the cytoplasm while the nucleus shows normal or no activity, (Figs. 1C and 2B).

At about the 7th day of the infection the lesions are larger, scattered throughout the liver, and tending to become confluent. Each lesion is a circular area of glycogen-deficient cells surrounding an area of pink-staining material. Haematoxylin and eosin sections show necrosis of many cells in the pink-staining area. Alkaline phosphatase activity is seen in the cytoplasm of cells at the periphery of the necrotic areas, and cells containing inorganic phosphate occur in the necrotic areas, but tend to disappear from the centre of large necrotic areas.

When both feet are injected, particularly if the infection runs a rapid course, the changes tend to be more diffuse. Cells deficient in glycogen occupy a much wider area or may be scattered throughout the section. In more advanced infections of this type, there are areas of necrosis but these are small in proportion to the total area of glycogen deficient cells, (Fig. 3B.)

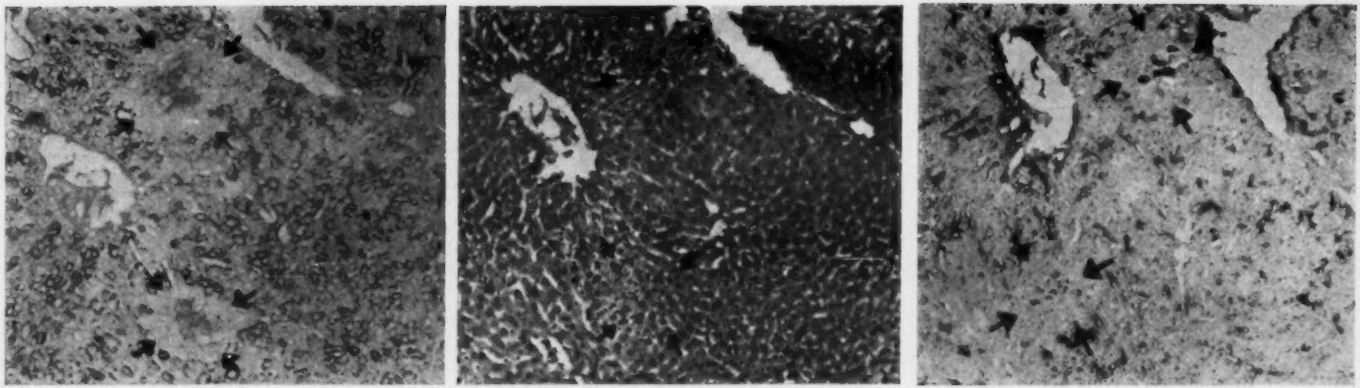

Fig. 1. Adjacent sections of liver from a mouse infected in one foot 6½ days before with Moscow strain.

Fig. 1A. Stained for glycogen by the periodate Feulgen method showing two areas of glycogen deficient cells, each having the pink staining material in the centre.

Fig. 1B. Section stained by Ehrlich's acid haematoxylin and eosin. The areas of necrosis correspond more closely to the pink staining material in Fig. 1A than to the area of glycogen deficient cells. The arrows show the size of the glycogen deficient areas shown in Fig. 1A. Necrosis is visible in the centre of the lesion, corresponding to the pink staining areas of Fig. 1A.

Fig. 1C. Stained for alkaline phosphatase activity. A few cells in each lesion show blackening in the cytoplasm. These areas correspond to the pink staining regions within glycogen deficient areas.

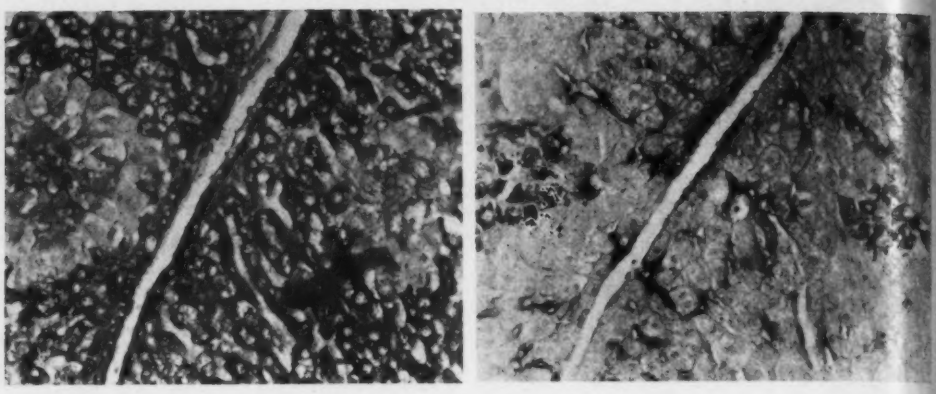

Fig. 2. Adjacent sections of liver from a mouse infected in one foot 7 days before.

Fig. 2A. Stained for glycogen. Two areas of glycogen deficient cells with the central pink staining material.

Fig. 2B. Stained for alkaline phosphatase. Well marked blackening is shown in the cytoplasm of many cells in the areas of glycogen deficiency shown in Fig. 2A. The blackening is made up largely of enzyme activity but some cells have preformed inorganic phosphate (as shown by the next section which is not reproduced here).

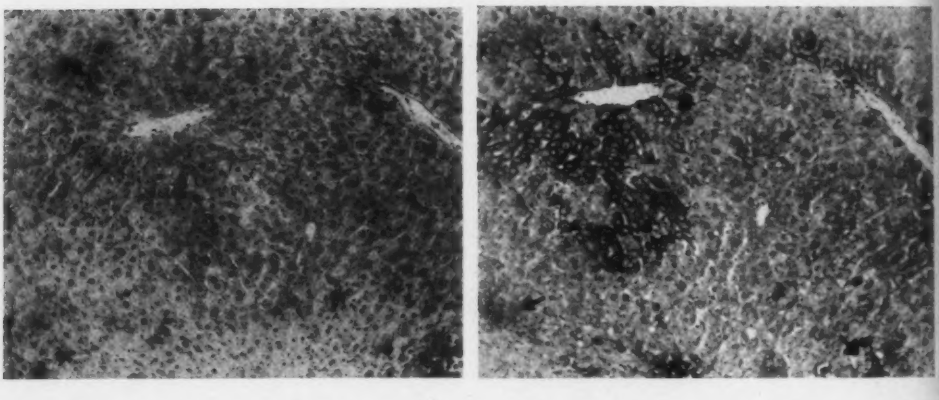

Fig. 3. Adjacent sections of liver from a mouse infected in both feet with Moscow strain 5 days before.

Fig. 3A. Stained for acid phosphatase. The blackening is much less than normal except for cells round and below the left hand vessel.

Fig. 3B. Stained for glycogen followed by Ehrlich's acid haematoxylin. The extent of glycogen filled cells (those with dark cytoplasm) is similar to the distribution of cells showing fair acid phosphatase activity in section 3A. There is a large area where morphologically intact cells are unable to synthesise glycogen. The arrows indicate small areas of necrosis.

The acid phosphatase may be depressed slightly before symptoms appear, but in sick animals it is markedly depressed. As is shown in Fig. 3A, at some stages of infection the areas of deficient acid phosphatase activity roughly coincide with areas of deficient glycogen synthesis. However, in rapidly advancing infections, the loss of glycogen precedes the loss of acid phosphatase, so the appearance in Fig. 3A and 3B is not always seen.

In the small series of mice infected with the Hampstead egg-passaged strain, no changes are seen until the 7–9th day and then only minor changes have been found. After starvation and glucose injections, no glycogen deficient areas such as those described above have been seen. Alkaline phosphatase activity is increased, but the distribution of the enzyme is the same as in normal mice. Acid phosphatase appears normal or slightly depressed. These changes appear to be reversible, and the mice recover.

DISCUSSION.

The failure of glycogen synthesis is the earliest and possibly the most important change.

An endocrine disturbance is unlikely to cause focal lesions of this kind, and the lesions are not altered when insulin is given. Preliminary measurements of oxygen consumption of liver slices in the presence of glucose show that in animals in which glycogen synthesis is impaired, there is no great fall in oxygen consumption. This is similar to the finding of Cohen and Anderson (1946) that in bacteria infected with bacteriophage, the oxygen consumption remained constant although bacterial multiplication was inhibited. Such infected bacteria are unable to synthesize bacterial enzymes as shown by Monod (1947). These findings have suggested that the energy and material produced by the bacterial cell are devoted to virus synthesis (Cohen, 1947).

The failure to synthesize glycogen is compatible with the hypothesis that energy derived from oxidation is devoted to synthesis of virus rather than of glycogen.

The few disturbances in livers infected with Hampstead strain may be due to the slower rate of growth of this virus, enabling the cell to supply building material and energy without irreversible damage to itself.

At the present time, it is difficult to separate primary from secondary changes in the cell, and a connected explanation of these findings is not possible.

The changes in phosphatase activities have been measured and agree with the histochemical findings. These results will be published separately.

SUMMARY.

The changes produced in livers of mice infected with large doses of ectromelia virus have been studied. The earliest change found in infections with the Moscow strain is a failure to synthesize glycogen. This is followed by the appearance of alkaline phosphatase in the cytoplasm, and a fall in acid phosphatase. Histological necrosis is visible shortly after alkaline phosphatase appears in the cytoplasm. In infections with the Hampstead strain, no loss of glycogen synthesis is found. The alkaline phosphatase activity is increased, but is normal in distribution, and the acid phosphatase activity falls slightly.

REFERENCES.

Cohen, Seymour S. and Anderson, T. F. (1946): J. exp. Med., 84, p. 511.
Cohen, S. S. (1947): Cold Springs Harbor Symposium on Quantitative Biology. Symp. quant. Biol., 12, p. 35.
Fenner, F. (1949): Austral. J. exp. Biol., 27, p. 31.
Gomori, G. (1939): Proc. Soc. exp. Biol. Med., 42, p. 23.
McManus, J. F. A. (1948): Stain Technology, 23, p. 99.
Monod, J. and Wollman, E. (1947): Ann. Inst. Pasteur, 73, p. 937.

INTERFERENCE BETWEEN INACTIVE AND ACTIVE INFLUENZA VIRUSES IN THE CHICK EMBRYO

I. QUANTITATIVE ASPECTS OF INTERFERENCE

By ALICK ISAACS[1] AND MARGARET EDNEY[2]

(From the Walter and Eliza Hall Institute of Medical Research, Melbourne).

(Accepted for publication 4th October, 1949.)

The interference phenomenon has been studied in regard to a number of plant, animal and bacterial viruses, but the mechanism of interference is not yet explained. The demonstration by Henle and Henle (1944) that influenza virus irradiated with U.V. light or inactivated by heat interferes with the multiplication of active influenza virus in the allantoic sac of the chick embryo has provided a useful means of analysing the phenomenon.

Inhibition by inactive (i.e. non-infective) virus of the multiplication of active influenza virus in the allantoic cavity might be due to a number of effects.

(1) Inactive virus might destroy enzymically the receptors required for the adsorption of active virus to the surface of the allantoic cells. This would be analogous to the action of the *V. cholerae* enzyme, RDE, which has been shown by Stone (1948) to prevent infection with influenza viruses in this way.

(2) Inactive virus might prevent adsorption of active virus to the surface of the allantoic cells by mechanical blocking of receptors (Ziegler, Lavin and Horsfall, 1944; von Magnus, 1946).

(3) Admission of an inactive virus particle into an allantoic cell might exclude the entry of further virus particles in a similar way to that in which entry of a sperm into the ovum excludes further sperms. This has been suggested as the mechanism of interference among bacterial viruses (Delbrück, 1945).[3]

(4) Inactive virus might compete with active virus for some intracellular substances essential for virus multiplication (Delbrück and Luria, 1942; Ginsberg and Horsfall, 1949).

In this paper and in that following we have studied some of the variables of the interference reaction and have attempted, in particular, to distinguish between the inhibition of virus multiplication which results from the use of

[1] Rockefeller Research Fellow.

[2] Francis Haley Research Officer, Department of Experimental Medicine, University of Melbourne.

[3] In a publication which appeared after this paper was sent to press, Delbrück (Colloques Internationaux du Centre National de Recherche Scientifique. Paris. 1949. VIII) has withdrawn this hypothesis.

(a) a large inoculum of inactive virus which may act by excluding active virus from the surface of the allantoic cells and (b) the *minimal* dose of inactive virus which will inhibit the multiplication of active virus. In the latter case, it appears that a different mechanism is involved.

Materials and Methods.

Virus Strains.

Influenza A. WSE—egg-adapted line of WS strain; PR8—American strain; MEL—Melbourne strain.

Influenza B. LEE—classical strain.

Mumps. American strain obtained by courtesy of Dr. J. F. Enders.

Newcastle Disease virus of fowls (NDV). Victorian strain of Albiston and Gorrie.

Normal Saline.

9·0 gm. sodium chloride in 1,000 ml. distilled water.

Citrate Saline.

20 gm. sodium citrate and 9·0 gm. sodium chloride in 1,000 ml. distilled water.

Borate Buffer.

Equal parts of M/20 sodium metaborate and M/5 boric acid (pH 8·5).

Calcium-Acetate Buffer Saline.

A sodium acetate-acetic acid buffer adjusted to pH 6·2 and containing 0·1 p.c. calcium chloride.

Fowl Red Cells.

Blood was obtained from the wing vein of adult white leghorn fowls. The cells were washed three times with normal saline and made up to a 1 p.c. suspension in normal saline.

Ovomucin.

Ovomucin was prepared from egg-white by the method described by Gottschalk and Lind (1949).

Haemagglutination Test (Modified from Hirst, 1942).

Serial twofold dilutions of virus (0·25 ml.) were prepared in normal saline and an equal volume of 1 p.c. fowl red cells was added to each dilution. The cells were allowed to settle at room temperature and readings were made by observing the pattern of sedimented cells as described by Burnet (1942). Partial, one plus (+), agglutination was taken as the end-point and the least amount of virus which will produce one plus (+) agglutination is referred to as one agglutinating dose (A.D.).

Infectivity Titrations of Active Virus.

Serial ten-fold dilutions of virus were prepared in 10 p.c. horse-serum saline. 0·05 ml. volumes were inoculated suballantoically into 11- or 12-day chick embryos. After incubation for a further 42 hours at 35° C. the allantoic fluids were harvested and one drop added to 0·25 ml. of 1 p.c. fowl cells. The 50 p.c. infectivity end-point (ID_{50}) is the dilution of virus which will infect 50 p.c. of the inoculated eggs.

Inactivation of Virus.

Two methods of inactivating virus were used in these experiments—formolization and heating.

Formolization—0·02 ml. of a 10 p.c. solution of formaldehyde was added to 10 ml. of allantoic fluid virus which was incubated for 16 hours at 37° C.

Heating—allantoic fluid virus was heated for an hour at 56° C.

Stone (1949) has shown that heating undiluted allantoic fluid preparations of some virus strains does not result in complete loss of enzymic activity of the virus, which does, however, occur if the virus is heated in citrate-borate buffer at pH 8·5. A modification of her method was used in these experiments. To 6 parts of allantoic fluid virus were added 2 parts of 2 p.c. citrate-saline and 1 part of borate buffer. This mixture was then heated for one hour at 56° C.

Inactivated virus was generally used on the day of preparation.

Tests of Inactivation of Virus.

Preparations of inactivated virus were tested for infectivity in eggs by inoculating 11- or 12-day eggs suballantoically. 0·5 ml. and 0·05 ml. of undiluted fluid, and 0·05 ml. of a 1 in 10 dilution, were each inoculated into a group of four eggs. After 42 hours' incubation at 35° C. the fluids were harvested and tested for haemagglutinin. A fluid which showed no agglutinin in any of twelve eggs inoculated in this way was regarded as non-infective.

Tests of Enzymic Activity of Virus.

In early experiments, inactivated virus was tested for enzymic activity by the inhibitor-destroying titration of Stone (1949). Two-fold dilutions of virus were prepared in 0·25 ml. volumes of calcium-acetate saline, and to each was added an equal volume of a standard dilution of ovomucin (containing 5–10 inhibitory doses, each dose being capable of inhibiting 5 agglutinating doses of the appropriate indicator virus). After incubation for 1 hour at 37° C. the mixtures were heated at 65° C. for 30 minutes to destroy the virus haemagglutinin, then cooled to room temperature. One drop of the indicator virus containing 5 agglutinating doses was added to each tube, followed after 30 minutes at room temperature by 0·25 ml. of a 1 p.c. suspension of fowl cells. The cells were allowed to settle at room temperature the end-point being the "+" degree of partial agglutination. Presence of agglutination indicates destruction of inhibitor, and hence enzymic activity of the virus.

Stone (1949) showed that viruses which had developed the "indicator" state (sensitivity of the virus haemagglutinin to inhibition by ovomucin) had lost their enzymic activity. In later experiments with heated virus, therefore, development of "indicator" state was taken as evidence of the loss of enzymic activity. The inhibitor-sensitivity of the virus was tested by making serial dilutions of ovomucin (0·25 ml.) and adding an equal volume of 5 agglutinating doses of the virus. After 30 minutes at room temperature a third volume of 1 p.c. fowl cells was added, and the usual "+" end-point read after the cells had settled. The method is described by Burnet (1948).

Interference Experiments.

The general technique of an experiment will be described; where differences in technique were introduced they are described in the text. Groups of six 11- or 12-day embryos were inoculated suballantoically with inactive virus. If 0·5 ml. or greater volumes were injected, an equal amount of allantoic fluid was first withdrawn. The eggs were incubated for one hour at 37° C. and then inoculated again with 0·05 ml. of challenge virus containing approximately 100 ID_{50}. Controls were inoculated with challenge virus only. The eggs were then incubated for 42 hours at 35° C. when the allantoic fluids were harvested and titrated individually for their haemagglutinin content. The Tables in the text show the geometric mean haemagglutinin titres of the group of six eggs ± the standard errors. The standard error was calculated from the formula

$$S.E. = \frac{\sqrt{\frac{\Sigma x^2}{n-1}}}{\sqrt{n}}$$

Preparation of Virus Eluates.

Five p.c. of packed fowl red cells was added to infected allantoic fluid and kept for 30 minutes at 4° C. to allow adsorption of virus on to the cells. The supernatant fluid was removed and the cells washed in an equal volume of chilled saline. The cells were resuspended in half the original volume of normal saline and incubated for 5 hours at 37° C. to allow elution of the virus. The red cells were removed from the eluate by light centrifugation.

EXPERIMENTAL.

Type of Inactive Virus Used as Interfering Agent.

The relationship of enzymic activity of inactivated influenza virus preparations to their interfering properties was first investigated. A batch of pooled allantoic fluid of strain MEL was inactivated in two ways, by formaldehyde, and by heating at 56° C. for one hour in citrate-borate buffer. (See Methods.)

Formolized virus was found to have lost its infectivity but the haemagglutinin titre and enzymic action on ovomucin were not significantly diminished. The second treatment caused loss of infectivity, roughly a twofold drop in haemagglutinin titre and complete loss of enzymic action on ovomucin.

The two preparations were then compared for their interfering powers. 2,000 A.D. and 1,000 A.D. of each were injected into groups of six 11-day embryos, suballantoically. After an hour at 37° C. the eggs were again inoculated suballantoically with 100 50 p.c. infective doses (ID_{50}) of PR8. Controls without interfering virus were injected with PR8 only, as described in Methods. The geometric mean haemagglutinin titres of the harvested fluids are shown in Table 1, together with their standard errors.

TABLE 1.

The rôle of the virus enzyme in interference.

Interfering virus		Interfering dose		
Mode of inactivation	Enzymic activity	2,000 A.D.	1,000 A.D.	0-control
Formaldehyde	100 p.c.	$12 \pm 7{\cdot}4$	35 ± 45	210 ± 149
Heat	0 p.c.	$2 \pm 2{\cdot}5$	0	

Interfering virus: MEL. Challenge virus: PR8, 100 ID_{50}.
The values shown are geometric mean haemagglutinin titres ± standard errors.

It can be seen that heated MEL, which lacks enzymic action on ovomucin, was a more efficient interfering agent than formolized MEL. In a number of further experiments of this type it was found that heated MEL was a reliable interfering agent; generally 300–1,000 A.D. produced complete inhibition of multiplication of active virus and this is referred to as the *minimal interfering dose*. The *minimal interfering dose* of virus is defined as the least amount of interfering virus which will prevent the appearance of haemagglutinin in eggs injected with 100 infective doses of challenge virus. Owing to the normal variations in the size of the allantoic sac of different eggs and of the resulting

interference, a mean haemagglutinin titre of less than 10 in a group of six eggs was taken as equivalent to complete interference for practical purposes. The *minimal interfering dose* of formolized MEL was found to be 2,000–4,000 A.D. and different preparations of formolized MEL were found to vary considerably in their interfering action. Further experiments were therefore carried out mainly with heated MEL.

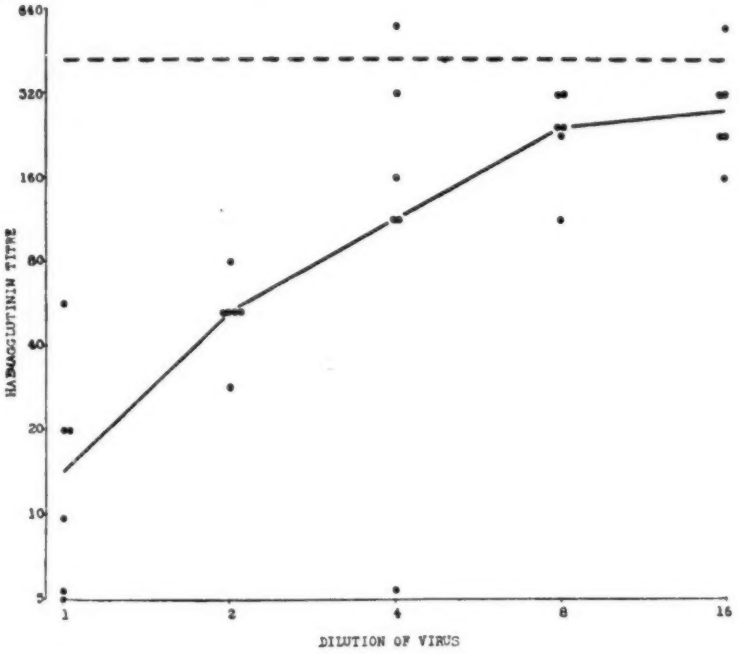

Fig. 1. Effect of variation in the dose of interfering virus.
Ordinate: Haemagglutinin titres of harvested eggs.
Abscissa: Dilution of interfering virus (heated MEL).
Each point represents the haemagglutinin titre of a single egg. The straight line is drawn through the geometric mean haemagglutinin titres. The dotted line is drawn through the titres of the control group.

Effect of Variations in the Concentration of Interfering Virus: Zone of Partial Interference.

The action of amounts of interfering virus less than the *minimal interfering dose* was investigated. A fresh preparation of heated MEL was diluted in saline 1 in 2 to 1 in 16. 0·5 ml. volumes of these dilutions were injected into groups of 11-day embryos suballantoically. After one hour's incubation at 37° C. the eggs were again inoculated with 100 ID_{50} of

active MEL. Controls were injected with 100 ID_{50} of active MEL only. The eggs were incubated for 42 hours at 35° C. and harvested and titrated as before. Table 2 shows the mean haemagglutinin titres of the groups inoculated with different dilutions of inactive virus.

TABLE 2.

Effect of variation in the dose of interfering virus.

Interfering dose (A.D.)	480	240	120	60	30	0-Controls
Mean haemagglutinin titres	15 ± 28·9	56 ± 20·8	120 ± 117	240 ± 30·2	300 ± 70·9	440 ± 100

Interfering virus: Heated MEL. Challenge virus: 100 ID_{50} of active MEL.

When the mean haemagglutinin titres are plotted against the dilution of interfering virus on a logarithmic scale it is seen that the points fall practically on a straight line (see Fig. 1). It is also clear from Table 2 that there is a wide zone of concentration of inactive virus which causes significant reduction in the haemagglutinin titre from that of the controls, i.e. a zone of partial interference.

Effect of Variations in the Concentration of Challenge Virus:

Experiments similar to the preceding one were carried out keeping the concentration of inactive virus constant and varying the concentration of virus in the challenge inoculum. A *minimal interfering dose* of virus for 100 ID_{50} of challenge virus was tested against a range of concentrations of challenge inoculum. It was soon found that within a wide range, 100 to 1,000,000 ID_{50}, there was only a slight difference in the interfering effect. However, when 10,000,000 to 100,000,000 ID_{50} of challenge virus was used the interference was largely overcome. An experiment illustrating this is shown in Fig. 2. Curve A shows the mean haemagglutinin titres of groups of 6 eggs injected with varying concentrations of active MEL only; as the amount of virus in the inoculum increases the mean haemagglutinin titre falls. This auto-interference phenomenon was described by Henle and Henle (1943). Curve B shows the titres of groups of 6 eggs similarly injected with MEL but also injected one hour previously with 960 A.D. of heated MEL. In each case, best-fitting curves have been drawn. Curve B shows a slow rise in haemagglutinin titre, becoming more steep as a concentration of 100,000,000 ID_{50}, i.e. undiluted allantoic fluid, is used. It is interesting to see that with undiluted active MEL, where the number of particles present in the inoculum does not differ greatly from the number present in the interfering inoculum the interfering effect of the heated MEL is negligible.

Effect of Length of Time Interval between the Injection of the Interfering and Challenge Viruses.

An interference experiment was carried out similar to those described above using constant quantities of interfering and challenge viruses and varying the time interval between the injections. In order to magnify any slight differences that might be found with different time intervals an amount of inactive virus was chosen less than the *minimal interfering dose*, i.e. one which would produce partial interference. Both formolized MEL and heated MEL

were tested as interfering agents; in each case the challenge inoculum was 100 ID_{50} of PR8 given at the appropriate time after the inactive virus. Controls were injected with 100 ID_{50} of PR8 only. The mean haemagglutinin titres in experiments with formolized MEL and heated MEL for different intervals of time are shown in Table 3.

TABLE 3.

Effect of variation in time interval between interfering and challenge viruses.

Interfering virus			Hours between injections					Control
Mode of inactivation	Enzymic activity	Dose used	0	1	3	6	24	
Formaldehyde	100 p.c.	1,280 A.D.	210 ± 55·8	160 ± 68·7	—	72 ± 24·5	100 ± 22·4	520 ± 82·5
Heat	0 p.c.	96 A.D.	210 ± 104·1	80 ± 60·0	112 ± 191	200 ± 123	—	448 ± 189

Interfering virus: MEL. Challenge virus: 100 ID_{50} of PR8.
The values shown are geometric mean haemagglutinin titres ± standard errors.

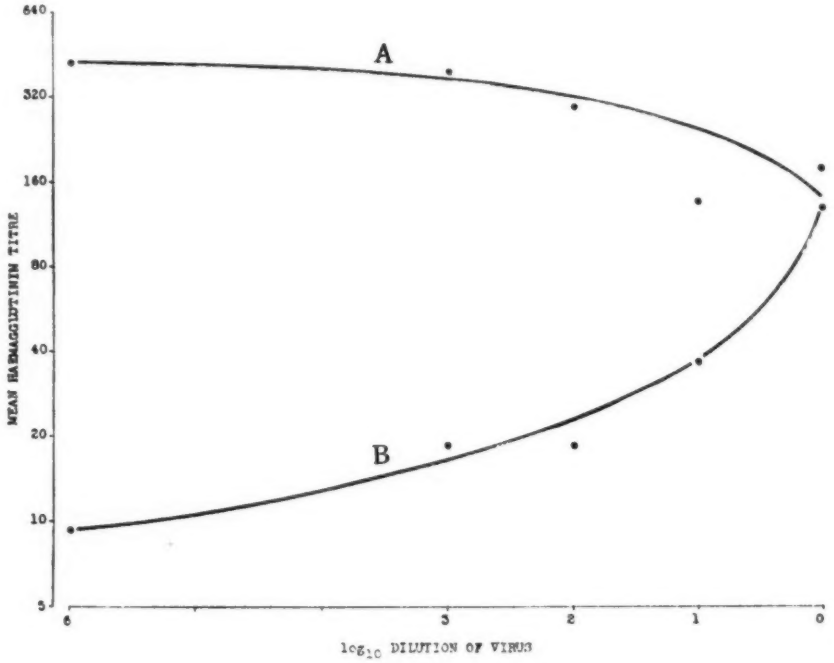

Fig. 2. Effect of variation in the dose of challenge virus.
Ordinate: Geometric mean haemagglutinin titre of group of six eggs.
Abscissa: Log_{10} dilution of challenge virus (MEL).
Curve A: Eggs inoculated with dilutions of challenge virus only.
Curve B: Eggs inoculated with 960 A.D. of heated MEL followed by inoculation of the same dilutions of challenge virus.

The Table shows that there are only slight differences in interference as a result of the different time intervals between the injections. It does appear, however, that formolized MEL caused greater interference when longer intervals of time were used. This did not apply to heated MEL in which a one-hour interval was found to be optimal, i.e. produced the greatest interference, and was used in further experiments.

Interference with Different Strains of Influenza Viruses.

With the viruses used, no marked quantitative differences have been found in interference using different strains. Stone (1949) has shown that by suitable treatment the majority of strains which she tested could be altered in such a way that they retained their haemagglutinating power but lost the power of enzymic elution from the cells.

Inactive viruses were prepared by heating allantoic fluid preparations of PR8 and LEE at 56° C. for an hour and tested for their interference against 500 ID_{50} of PR8. Controls were injected with 500 ID_{50} of PR8 only. Table 4 shows the extent to which they interfered with the multiplication of PR8. Heated PR8 appears to be a slightly less effective interfering agent than heated MEL or heated LEE but the differences are barely significant.

TABLE 4.

Action of minimal interfering dose of heated PR8, LEE and MEL.

Challenge virus: 500 ID_{50} of PR8.

Strain	Interfering virus: Enzymic activity	Interfering virus: Dose used	Mean haemagglutinin titres: Assay	Mean haemagglutinin titres: Control
PR8	nil	1,280 A.D.	30 ± 12·3	1,000 ± 128
LEE	nil	480 A.D.	8 ± 3·4	
MEL	nil	600 A.D.	13 ± 4·7	
Challenge virus: 100 ID_{50} of WSE.				
PR8	nil	600 A.D.	28 ± 12·8	580 ± 53·5
LEE	nil	960 A.D.	11 ± 6·6	
MEL	nil	960 A.D.	6 ± 4·9	

Table 4 (lower half) also shows a similar experiment with different preparations of the same inactive viruses; in this case 100 ID_{50} of WSE was used as challenge virus and controls were injected with 100 ID_{50} of WSE only. The two experiments gave essentially similar results.

In further experiments, it was found that the *minimal interfering dose* of heated LEE was effective in preventing the multiplication of 100 ID_{50} of homologous or heterologous challenge viruses. A result of this type is shown in Table 5.

The Table shows the mean haemagglutinin titres of groups of eggs inoculated with 0·5 ml. of heated LEE followed after an hour by an inoculation of 100 ID_{50} of challenge virus. The control eggs received the challenge virus only.

TABLE 5.

Action of minimal interfering dose of heated LEE on multiplication of PR8, WSE, MEL, LEE, NDV and Mumps.

Eggs injected with	Strain of challenge virus					
	PR8	WSE	MEL	LEE	NDV	Mumps
Heated LEE and challenge virus	0	3 ± 1·7	12 ± 9·6	0	8 ± 4·2	0
Challenge virus only	1,000 ± 98·4	224 ± 100	510 ± 131	144 ± 90·4	140 ± 61·8	15 ± 16

Interfering virus: 0·5 ml. heated LEE. Challenge viruses: $100ID_{50}$ of respective strain. Mumps—10^{-1} dilution of allantoic fluid.
The values shown are geometric mean haemagglutinin titres ± standard errors.

In addition to the four strains of influenza virus tested as challenge viruses, strains of Newcastle disease (NDV) and mumps viruses were also included. Mumps virus was grown in the allantoic cavity of eight-day embryos which were then incubated for five days at 35° C. before harvesting. Otherwise the details of the experiments were the same as those with influenza viruses, the haemagglutinin level of harvested fluids being taken as the criterion of virus multiplication. It can be seen (Table 5) that multiplication of both viruses was inhibited by a *minimal interfering dose* of inactive LEE. It was interesting to note that inactive LEE inhibited production of NDV in the allantoic sac but did not prevent the lethal action of the virus on the embryo—presumably the spread of the NDV was not prevented, i.e. the inactive LEE had a local action only. The low mean haemagglutinin titre and high standard error found in the control group in the experiment with mumps virus are due to the known difficulty in infecting 100 p.c. of a group of eggs inoculated with this virus. Actually, 15 out of 22 eggs showed production of haemagglutinin compared with 0 out of 11 in the group inoculated with inactive LEE and mumps virus. The results are at variance with those of Ginsberg and Horsfall (1949) who reported no interference between active influenza and mumps viruses in the allantoic sac of the chick embryo.

These results suggest that there are no marked strain differences in the ability to interfere with the multiplication of any of the viruses investigated. Also, inactive forms of PR8, MEL and LEE gave values for the *minimal interfering dose* within a narrow range.

Identification of Inactive Virus as Interfering Agent.

Throughout these experiments the assumption has been made that the inactive virus present in these preparations is the interfering agent. This assumption has been based on the work of Henle and Henle (1945), who showed the identity of the interfering agent and the virus particle, in experiments using centrifugation and neutralization by specific antiserum.

Since the inactive virus used in these experiments differed slightly from that used by other workers, two formal experiments were carried out to confirm this assumption. In the first experiment, an allantoic fluid preparation of MEL virus was adsorbed on to fowl red cells and eluted into normal saline. The eluate was then heated in the same way as for MEL allantoic fluid. The heated MEL eluate produced inhibition of multiplication of active MEL and gave a *minimal interfering dose* of the same order as standard preparations of heated

MEL. In the second experiment a preparation of LEE allantoic fluid was divided into two portions which were heated for one hour, one at 56° C. and the other at 60° C. Heating at 56° C. caused a twofold reduction in haemagglutinin titre and 0·5 ml. of this (HA titre 240) produced marked interference with the multiplication of active MEL. Heating at 60° C. caused complete destruction of the haemagglutinin and 0·5 ml. volumes caused no interference with the multiplication of MEL.

Thus, the interfering agent, like the haemagglutinin can be adsorbed by and eluted from fowl red cells and is stable after heating for an hour at 56° C. but not at 60° C.

DISCUSSION.

In a series of studies, the Henles and collaborators have shown that the allantoic sac of the chick embryo is a useful site for the study of the interference phenomenon. By the use of inactivated virus as interfering agent it becomes possible to remove one variable from the reaction and to study the phenomenon quantitatively. In assessing the results of experiments with this technique it is therefore essential to show that the interfering virus is non-infective under the experimental conditions used. This requires tests of infectivity of undiluted interfering virus and of the same material diluted past the *minimal interfering dose*, in order to avoid "auto-interference". All interfering agents used in these studies were tested for infectivity in this way and only materials non-infective by these tests were used.

Experiments in which a comparison was made between formolized MEL and heated MEL (heated at 56° C. for an hour in citrate-borate buffer pH 8·5) as interfering agents have shown that the latter is more efficient. It is clear from these results that enzymic action of the virus is not essential for interfering activity. Formolized MEL and heated MEL were also compared in regard to the optimal time interval between the injection of interfering and challenge viruses in order that the greatest interference should result. In contrast to heated MEL, formolized MEL was found to show significantly greater interference with longer intervals of time. It is probable that this difference can be related to the enzymic activities of the two preparations. In the following paper it will be shown that large amounts of inactive virus are taken up by the allantoic cells, by a process which Fazekas de St.Groth (1948) has termed "viropexis". Heated MEL, lacking enzymic activity, would be rapidly adsorbed to the surface of the allantoic cells from which enzymic elution would not be possible. Formolized MEL is capable of enzymic elution from the surface of the allantoic cells, however, and hence it might be expected that after a longer interval of time a greater amount of effective interfering virus would be taken up by the allantoic cells by "viropexis".

The *minimal interfering dose* for heated PR8, heated LEE and treated MEL was found to be about 0·5 ml. of undiluted allantoic fluid or roughly 500–1,000 agglutinating doses. Freshly harvested allantoic fluid of these strains has usually an infectivity of 10^{-8} to 10^{-9}, but it is difficult to calculate

how many virus particles would be present in the *minimal interfering dose* until it is known how many particles make up one infective dose. Similarly, it is known that the number of cells lining the allantoic sac is between 10^8 and 10^9 (Hoyle, 1948) but it is not known how many of these cells are susceptible to infection with influenza viruses. Any attempt at calculation, by this method, of the average number of virus particles per allantoic cell required to initiate interference is thus confronted with two unknown quantities.

Another method of trying to answer this question is to examine the effect produced by injections of inactive virus less than the *minimal interfering dose*. If it is assumed that one inactive virus particle is capable of preventing multiplication of active virus in an allantoic cell, one can calculate from the Poisson distribution the average number of inactive virus particles per cell required to block 99 per cent. of the susceptible cells. This would happen when there is an average of four particles per allantoic cell, provided contacts between virus particles and allantoic cells occur at random. Hence the least amount of inactive virus required to produce "complete (99 p.c.) interference" on this assumption is an inoculum containing an average of four particles per allantoic cell. If it is further assumed that the least amount of virus capable of causing any significant degree of interference would be enough to provide one inactive virus particle for half the allantoic cells, this would be roughly one-eighth of the *minimal interfering dose*, an amount which is in close agreement with the experimental findings. However, this evidence is insufficient for accepting these assumptions, since the zone of partial interference might equally well result, if a number, x, of inactive particles produced complete interference in an individual cell and $\frac{x}{2}$ and $\frac{x}{4}$ particles produced a reduction in the "burst" size of individual cells (Henle, Henle and Rosenberg, 1947). There is not yet sufficient evidence for deciding between these hypotheses.

In view of the number of possible sources of error in these experiments, the results have shown a high degree of reproducibility. However, there is no indication from these or other experiments, of the degree of variation of the allantoic cells in their reactions to the presence of active or inactive virus. Hence, the final haemagglutinin titre produced following infection of a single egg may represent the average results of a very wide scatter in the reactions of individual cells. The arguments used in this discussion have been based on the final results of an experiment, and therefore on the average behaviour of individual allantoic cells.

It is interesting to note that the interfering action of a *minimal interfering dose* can be overcome by increasing the amount of virus in the challenge inoculum. The shape of the curve (curve B, Fig. 2) suggests a competition between the active and inactive viruses for a substance or substances essential for virus multiplication. This point will be discussed further in Paper II when the site of the interference process is considered.

SUMMARY.

A study was made of interference by inactive influenza virus with the multiplication of active influenza viruses in the allantoic sac of the chick embryo.

Heated virus lacking enzymic activity was a better interfering agent than formolized enzymically active virus.

300–1,000 A.D. of heated virus (MEL) was the least amount which produced complete inhibition of 100 ID_{50} of active virus (*minimal interfering dose*).

One-eighth of the *minimal interfering dose* produced significant inhibition of multiplication of 100 ID_{50} of active virus, i.e. partial interference.

The *minimal interfering dose* showed no significant inhibition of multiplication of 100,000,000 ID_{50} of active virus.

The *minimal interfering dose* of strains PR8, MEL and LEE was of the same magnitude.

A *minimal interfering dose* of heated LEE inhibited multiplication of WSE, PR8, MEL and LEE influenza viruses, and strains of Newcastle disease and mumps viruses.

REFERENCES.

Burnet, F. M. (1942): Austral. J. Sci., 5, p. 5.
Burnet, F. M. (1948): Austral. J. exp. Biol., 26, p. 371.
Delbrück, M. and Luria, S. E. (1942): Arch. Biochem., 1, p. 111.
Delbrück, M. (1945): J. Bact., 50, p. 151.
Fazekas de St.Groth, S. (1948): Nature, 162, p. 294.
Ginsberg, H. S. and Horsfall, F. L. Jr. (1949): J. exp. Med., 89, p. 37.
Gottschalk, A. and Lind, P. E. (1949): Brit. J. exp. Path., 30, p. 85.
Henle, W. and Henle, G. (1943): Science, 98, p. 87.
Henle, W. and Henle, G. (1944): Amer. J. med. Sci., 207, p. 705.
Henle, W. and Henle, G. (1945): *Ibid.*, 210, p. 369.
Henle, W., Henle, G. and Rosenberg, E. B. (1947): J. exp. Med., 86, p. 423.
Hirst, G. K. (1942): *Ibid.*, 75, p. 49.
Hoyle, L. (1948): Brit. J. exp. Path., 29, p. 390.
Stone, J. D. (1948): Austral. J. exp. Biol., 26, p. 49.
Stone, J. D. (1949): *Ibid.*, 27, p. 337.
von Magnus, P. (1946): Arkiv. für Kem, Mineral. och Geologi, 24B, p. 7.
Ziegler, J. E., Lavin, G. I. and Horsfall, F. L. (1944): J. exp. Med., 79, p. 379.

INTERFERENCE BETWEEN INACTIVE AND ACTIVE INFLUENZA VIRUSES IN THE CHICK EMBRYO

II. THE SITE OF INTERFERENCE

By ALICK ISAACS[1] AND MARGARET EDNEY[2]

(From the Walter and Eliza Hall Institute of Medical Research, Melbourne).

(Accepted for publication 4th October, 1949.)

In considering the site of action of inactive interfering virus it is necessary to define the amount and the enzymic state of the virus used. Interference produced by injection into eggs of a large inoculum of inactive virus possessing enzymic activity might result partly from enzymic destruction of receptors, partly from mechanical blocking of receptors and partly from other means. In the present experiments, we have examined the site of action of a *minimal interfering dose* of heated virus, i.e. the least amount of inactive virus which will inhibit multiplication of active virus, as described in the preceding paper. This virus lacks enzymic activity so that enzymic destruction of receptors is excluded from the possible causes of its interfering action.

A second possibility is that the inactive virus blocks all receptors, thus preventing entry of live virus into the cells. In order to exclude this hypothesis, it is necessary to show that the allantoic sac is capable of adsorbing a greater quantity of virus than the *minimal interfering dose*. Henle, Henle and Kirber (1947) and Ginsberg and Horsfall (1949) carried out experiments to find out the adsorptive capacity of the allantoic surface, by injecting virus into the allantoic sac and estimating the haemagglutinin titre of samples withdrawn after various intervals of time. These authors noted a reduction in the haemagglutinin titre of the inoculated virus. However, it is known that normal allantoic fluid contains an inhibitor of influenza virus haemagglutination (Svedmyr, 1947 and 1948; Hardy and Horsfall, 1948). Hence, some of the apparent disappearance of virus from the allantoic fluid described by the above authors was probably due to inhibition of agglutination by the allantoic fluid inhibitor.

A third possibility is that following the taking up of inactive virus by the allantoic cells, live virus is adsorbed to, but is excluded from entering, the cells. This is similar to the "penetration" hypothesis described by Delbrück (1945) for bacteriophage. The experiments reported in this paper suggest that when the *minimal interfering dose* of heated virus is used, inhibition of

[1] Rockefeller Research Fellow.

[2] Francis Haley Research Officer, Department of Experimental Medicine, University of Melbourne.

virus multiplication is not caused by either of these mechanisms and entry of live virus into the allantoic cells is not prevented.

Materials and Methods.

Most materials and methods are described in the preceding paper.

RDE.

An extract of the 4Z strain of *V. cholerae* containing the receptor-destroying enzyme (RDE) was prepared as described by Burnet and Stone (1947).

Antiserum.

An antiserum was prepared by bleeding a ferret 10 days after intranasal inoculation with LEE allantoic fluid. The serum had an antihaemagglutinin titre of 1,000 against 5 agglutinating doses of LEE virus.

Titration of Inhibitor in Allantoic Fluid.

Serial dilutions (0·25 ml.) of allantoic fluid were prepared in normal saline. An equal volume of heated MEL (Paper I, Methods) containing 5 agglutinating doses of virus, was added, and kept for 30 minutes at room temperature. A third volume of 1 p.c. fowl cells was added and these were allowed to settle at room temperature. The least amount of inhibitor causing partial (+) agglutination was taken as the end-point.

Titration of Antihaemagglutinin.

Serial dilutions (0·25 ml.) of antiserum were prepared in normal saline and an equal volume of 1 p.c. fowl cells was added. A third volume containing 5 A.D. of LEE virus was added, mixed, and the cells allowed to settle. The test was read in the same way as for the inhibitor titrations.

EXPERIMENTAL.

The Inhibitor in Normal Allantoic Fluid.

When a large amount of virus is injected into the allantoic cavity of normal 11-day eggs and samples of allantoic fluid withdrawn at intervals, it is found that there is an immediate sharp drop in haemagglutinin titre, followed by a slow decline. The immediate drop is more marked with heated than with active virus and is due to the inhibition of haemagglutination by the inhibitor in normal allantoic fluid. An attempt was made to remove the inhibitor by washing out the allantoic cavity. The allantoic fluid was drained as completely as possible and 5 ml. of normal saline (37° C.) were injected. This was repeated three times. The allantoic fluid and washings were then titrated for their content of inhibitor of agglutination against 5 agglutinating doses (A.D.) of heated MEL. The results of a typical experiment are shown in Table 1.

It can be seen that the first and second washings produced a 4-fold reduction in inhibitor titre, but that the third produced no further reduction. It was thus found to be impracticable to remove by this means all inhibitor for agglutination by heated MEL.

TABLE 1.

Attempted removal of inhibitor from the allantoic cavity by washing.

Sample	Inhibitor titre
Allantoic fluid	200
1st washing	48
2nd washing	12
3rd washing	12

Adsorption of Inactive Virus in the Allantoic Cavity.

In order to follow the adsorption of inactive virus in the allantoic sac, it was necessary to reduce as much as possible the effect of the inhibitor in normal allantoic fluid. This was done by first washing out the allantoic cavity with saline and then treating all samples with *V. cholerae* filtrate containing the receptor-destroying enzyme, RDE, which destroyed all remaining inhibitor.

The allantoic cavity of normal 11-day embryos was washed with three 5 ml. volumes of normal saline. The third washing was then removed and replaced by 5 ml. of inactive virus. The eggs were rotated to mix the virus, and samples were removed immediately, and after various periods of time. Part of each sample was titrated for its haemagglutinin content directly. To the other portion of each sample *V. cholerae filtrate* (RDE) was added in a concentration of 1 in 5. This was incubated for thirty minutes at 37° C. and then titrated for its haemagglutinin content in 2 p.c. citrate-saline. Table 2 shows the results in a representative experiment. In this case, the inoculum was 5 ml. of heated MEL, haemagglutinin titre 240 (4,800 A.D.). The haemagglutinin titre of each sample is shown, untreated and following treatment with RDE.

TABLE 2.

Adsorption of inactive virus in the allantoic cavity.

Time of sampling	Haemagglutinin titres	
	Untreated	RDE-treated
1–5 minutes	16	85
1 hour	2	72
2 hours	<2	48
5 hours	<2	27

Inoculum—5 ml. of heated (inactive) MEL, haemagglutinin titre 240 (total 4,800 A.D.).

The differences in titre in the two columns of Table 2 reflect the content of inhibitor remaining in the allantoic fluid after washing of the cavity. The inhibitory titres of the washings shown in Table 1 belong to the same experiment as that in Table 2. The inhibitory titre of the final washing is 12,

measured against 5 agglutinating doses (A.D.) of heated MEL. Hence, sufficient inhibitor was present per unit volume to inhibit the agglutination of 60 A.D. of heated MEL. Table 2 shows that the differences between the titres in the first and second columns approximate closely to 60 A.D. in the immediate and one-hour samples. It appears therefore, that following treatment of the samples with RDE, the inhibitor is destroyed and that it is then possible to obtain an accurate indication of the haemagglutinin titre of the samples.

It can also be seen from Table 2 that approximately 80 p.c. of the inoculum (4,000 agglutinating doses) had disappeared from the allantoic fluid in 5 hours, when corrections for dilution are made. In further experiments in which samples were taken after 24 hours, it was found that 5 ml. of heated LEE, haemagglutinin titre 640, were completely adsorbed. This means adsorption of 12,800 agglutinating doses of inactive virus, an amount which is greatly in excess of the *minimal interfering dose* of inactive virus (300–1,000 A.D.).

Action of RDE in Preventing Adsorption of Inactive Virus.

Stone (1948) showed that suballantoic injection of RDE into eggs prevented adsorption of active virus. The following experiment shows that RDE has the same effect with inactive virus.

Two normal 11-day eggs were washed thrice with 5 ml. saline; 1 ml. of RDE was added to the third saline injection of one egg and the eggs were incubated for 90 minutes at 37° C. to allow the RDE to act. The allantoic fluids were then removed and 5 ml. of heated PR8 were injected into each egg. Samples were withdrawn immediately after mixing, and hourly thereafter, and were titrated for their haemagglutinin content. The samples from the control egg were treated with 1 in 5 RDE *in vitro*, as in the last experiment, in order to remove residual inhibitor. The results are shown in Table 3.

TABLE 3.

Adsorption of inactive virus in allantoic cavity of normal and RDE-treated eggs.

Time of sampling	Haemagglutinin titres	
	Normal egg	RDE-treated egg
1–5 minutes	100	120
1 hour	40	100
2 hours	20	80
3 hours	20	100
4 hours	15	160

It can be seen that in the control egg adsorption of heated PR8 occurs as in the previous experiment. In the egg treated with RDE adsorption of virus is prevented, as shown by the constant haemagglutinin titre of samples throughout the experiment.

"Viropexis" of Inactive Virus.

Fazekas de St.Groth (1948) showed that inactive virus injected into the allantoic sac was first adsorbed to the surface of the allantoic cells and was then taken into the cells by a process which he termed "viropexis". The presence of inactive virus on the surface of the allantoic cells was determined in two ways (a) virus could be released from the surface of the cells by the receptor-destroying enzyme, RDE (b) when specific antiserum was injected into the allantoic sac, combination of virus and antiserum was shown by a reduction in the antihaemagglutinin titre of the serum. Fazekas de St.Groth showed by means of both techniques that following the injection of inactive virus into the allantoic sac, the amount of virus which could be demonstrated on the surface of the allantoic cells decreased greatly over a period of 3 hours. The same techniques were therefore used, to see whether the allantoic cells would take up the greater amounts of inactive virus which have been used in the present interference experiments.

The allantoic cavity of 11-day embryos was washed thrice with 5 ml. normal saline and the final washing was replaced by 5 ml. heated LEE (4,800 A.D.). The eggs were incubated for 18 hours at 35° C. when the allantoic fluids were removed. No haemagglutinin was detected in these fluids following treatment *in vitro* with 1/5 RDE. The eggs were divided into two series. In one, they were injected allantoically with 5 ml. of 1/5 RDE and incubated for an hour at 37° C. Samples were then removed and titrated for haemagglutinin, but in no case was virus recovered. In the second series, the eggs were injected with 5 ml. of a 1/100 dilution of a ferret antiserum to LEE virus. After 30 minutes' incubation at 37° C. the antiserum was withdrawn and titrated for its antihaemagglutinin content. No significant reduction in antihaemagglutinin content was found.

Both these results suggest, therefore, that the large inoculum of inactive virus (4,800 A.D.) was completely taken up by the allantoic cells in 18 hours.

Adsorption of Active Virus in Normal Eggs and in Eggs Previously Injected with a Minimal Interfering Dose *of Inactive Virus.*

It was of interest to find out whether, following injection of a *minimal interfering dose* of inactive virus, there was any difference from normal eggs in the rate of adsorption of active virus injected subsequently. Henle and Rosenberg (1949) have reported that following injection of 1,000 to 10,000 ID_{50} of active virus into the allantoic sac of normal 11-day embryos, 63–90 p.c. of the virus was adsorbed. We have found a similar degree of adsorption when a large inoculum was given to normal 11-day eggs. Thus, when 256–512 agglutinating doses (approximately 10^9 ID_{50}) of active PR8 were injected suballantoically, 66–75 p.c. of virus was adsorbed in one hour, as measured by haemagglutination tests of samples treated with RDE. A

variation was noted with different strains, since lower values (10–50 p.c.) were found in similar experiments with active MEL. When 10^7 ID_{50} of active MEL were injected suballantoically, there was a 90 p.c. (or one $\log_{10}$ unit) adsorption, as measured by the less accurate infectivity titration. However, when 100 ID_{50} of MEL were injected suballantoically, the adsorption found was less than one $\log_{10}$ unit. This paradoxical result does not appear to be due to the presence of an inhibitor in normal allantoic fluid, as the same result was found when the experiment was carried out in washed eggs.

The adsorption of 100 ID_{50} active MEL in eggs previously injected with a *minimal interfering dose* of treated MEL was of the same order as in normal eggs. Usually, adsorption was slightly more marked in "interfered" eggs, but owing to the error of the method the differences may not be significant. The results of a representative experiment are shown in Table 4. Two 11-day embryos were used. The allantoic cavities were drained and washed out thrice with 5 ml. normal saline. To the third 5 ml. volume of saline of one egg, 0·5 ml. of heated MEL (640 A.D.) was added. After 90 minutes at 37° C. the allantoic fluids of both eggs were removed and replaced by 5 ml. of saline containing approximately 100 ID_{50} of active MEL. The eggs were incubated at 35° C. for two hours, when samples of allantoic fluid were removed and titrated in eggs, using tenfold dilutions. The results of the infectivity titrations of the inoculum and the samples from the normal and "interfered" egg are shown in Table 4.

TABLE 4.

Adsorption of 100 ID_{50} of MEL virus in normal and "interfered" allantoic cavity.

Dilution	Infectivity of fluids from		
	Inoculum	Control egg	"Interfered" egg
10^0	—	5/5	3/4
10^{-1}	5/5	5/5	3/5
10^{-2}	3/5	1/5	0/5
10^{-3}	0/5	—	—

The numerator gives the number of eggs showing growth of virus; the denominator shows the number of eggs inoculated.

The results in Table 4 show that adsorption was slightly greater in the egg previously injected with a *minimal interfering dose* of heated MEL than in the normal egg and a similar result was found in six out of nine experiments. Adsorption of equal amounts of virus in the two groups was found in three out of nine experiments. However, it is clear that following injection of a *minimal interfering dose* of heated MEL there was no inhibition of adsorption of 100 ID_{50} of active MEL.

It was possible that in an experiment similar to the above in "interfered" eggs, the active MEL was adsorbed to the surface of the allantoic cells but was unable to enter the cells. In order to test this, experiments were carried out similar to that shown in Table 4. Following the removal of a sample of allantoic fluid to titrate its content of virus, the eggs were injected with 1 ml. of *V. cholerae* filtrate (RDE) and incubated for 30 minutes at 37° C. The RDE would remove active virus present on the surface of the cells. A second sample was then removed and titrated for its infectivity. There was insufficient RDE or inactive virus present in this sample to inhibit the multiplication of active virus. It was found that the content of virus in samples following injection of RDE into eggs was approximately the same as that in samples taken before RDE was injected. There was thus no evidence that the active virus adsorbed on to the surface of the allantoic cells was prevented from entering the cells.

DISCUSSION.

The findings described in this paper suggest that inhibition of multiplication of live virus by inactive virus is not the result of exclusion of active virus from entering the allantoic cells. The evidence in favour of this statement can be summarized as follows:

(1) Interference can be produced by inactive virus lacking enzymic activity. Hence destruction of receptors by enzymic action of the virus is not the mechanism of interference in these experiments.

(2) The adsorptive capacity of the allantoic sac for inactive virus is considerably greater than the *minimal interfering dose*, which would thus be capable of blocking only a fraction of the available receptors on the allantoic cells.

(3) The cells lining the allantoic sac are capable of taking up ("viropexis") in 18 hours, at least ten times the *minimal interfering dose* of inactive virus. This provides strong evidence that the allantoic cell is capable of taking up more than one virus particle. The hypothesis put forward for bacteriophage by Delbrück (1945) that following entry of one virus particle, the cell is unable to take up further particles, would therefore not account for interference by inactive influenza virus in the allantoic sac. The fact that amounts of inactive virus greater than the *minimal interfering dose* are taken into the allantoic cells by "viropexis" is also strong evidence in favour of the intracellular site of the interference processes.

(4) Following injection of a *minimal interfering dose* of inactive virus there was no inhibition of the adsorption to allantoic cells of active virus injected subsequently.

(5) Following injection of a *minimal interfering dose* of inactive virus there was no inhibition of the uptake by allantoic cells of active virus injected subsequently.

These results lead to the conclusion that interference is an intracellular phenomenon, which might result from (a) nonspecific damage to the allantoic cells by inactive virus, rendering the cells unsuitable for multiplication or from (b) competition by inactive virus with active virus for cell constituents essential for virus multiplication. In the previous paper it was shown that the effect of a *minimal interfering dose* of inactive virus could be overcome by increasing the dose of challenge virus. This result favours the hypothesis of competition for vital cell constituents. Examination of the curve (previous paper, Fig. 2, curve B) shows that it does not rise uniformly as the concentration of challenge inoculum increases, but that it rises slowly at first and more steeply when 10^8 ID_{50} of challenge virus are injected. This is the type of result one would expect if interference resulted from competition within the allantoic cells for cell constituents essential for multiplication. Thus, when inactive and active virus particles are competing for essential substances within a given allantoic cell, the inhibitory effect of the inactive virus would be overcome when the ratio of active particles to inactive particles *per allantoic cell* is increased. As yet, there is no indication of the nature of any essential substances.

SUMMARY.

The allantoic sac of washed eggs was found to adsorb 12,800 A.D. of heated LEE, an amount greatly in excess of the *minimal interfering dose*. Adsorption of inactive virus was inhibited by pre-treatment of eggs with RDE.

Inactive virus in excess of the *minimal interfering dose* was taken up ("viropexis") by the allantoic cells.

Following injection of a *minimal interfering dose* of inactive virus there was no inhibition of adsorption of active virus to the allantoic cells or of uptake of active virus by the allantoic cells.

These findings point to an intracellular site of the interference process, and it is suggested on the basis of experiments in Paper I, that interference results from competition by inactive virus with active virus for cell constituents required for virus multiplication.

Acknowledgment. We are deeply indebted to Professor F. M. Burnet for his advice and criticism.

REFERENCES.

Burnet, F. M. and Stone, J. D. (1947): Austral. J. exp. Biol., 25, p. 227.
Delbrück, M. (1945): J. Bact., 50, p. 151.
Fazekas de St.Groth, S. (1948): Nature, 162, p. 294.
Ginsberg, H. S. and Horsfall, F. L., Jr. (1949): J. exp. Med., 89, p. 37.
Hardy, P. H. and Horsfall, F. L., Jr. (1948): *Ibid.*, 88, p. 463.
Henle, W., Henle, G. and Kirber, M. W. (1947): Amer. J. med. Sci., 214, p. 529.
Henle, W. and Rosenberg, E. B. (1949): J. exp. Med., 89, p. 279.
Stone, J. D. (1948): Austral. J. exp. Biol., 26, p. 49.
Svedmyr, A. (1947): Arkiv für Kem., Mineral. och Geologi, 248, p. 1.
Svedmyr, A. (1948): Brit. J. exp. Path., 29, p. 295.

THE ACTION OF YOHIMBINE AND POTASSIUM ON CONDUCTION AND CONTRACTION IN AMPHIBIAN STRIATED MUSCLE

by I. A. N. McCALLUM, F. H. SHAW AND W. T. AGAR[1]

(From the Department of Physiology, University of Melbourne).

(Accepted for publication 11th October, 1949.)

Following an investigation of the action of yohimbine on amphibian sciatic nerve (MacCallum and Shaw, 1949), it was decided to subject amphibian striated muscle to the same conditions, in order to observe any action it might have on conduction and contraction. If conduction were abolished, as it is in nerve, was the ability of the muscle to contract also abolished? The work of several investigators, including Ramsey and Street (1941), indicated that stimulation with massive electrodes may cause a muscle to contract, even when conduction had been abolished—this provides a test for contractility independent of conduction.

METHOD.

A paraffin wax bath was constructed, with a central recess 9 cm. × 1 cm. In the walls of the recess were embedded two horizontal silver plates, 5·5 cm. in length, and of gauge 24 in thickness, with their edges 6 mm. above the bottom of the recess and flush with its side walls. Tension changes were recorded by a moveable arm attached to a sensitive wire strain gauge, so that any changes in tension on the hook produced corresponding electrical variations from the gauge.

The sartorius from the toad, *Bufo marinus*, was carefully dissected out and attached, by coarse thread tied to the tendinous tibial end, to the adjustable support. The aponeurotic pelvic end of the muscle was attached by coarse thread to the hook connected with the strain gauge. Two stimulating electrodes of silver wire were placed in light contact with the muscle, transversely across its pelvic end. Two pick-up electrodes of silver wire one centimetre apart were placed in light contact with the tibial end of the muscle.

The strain gauge was connected through a direct coupled amplifier to an oscilloscope, and the pick-up electrodes were connected through a second direct-coupled amplifier to another oscilloscope. The horizontal plates of both oscilloscopes were connected to triggered time bases, which also modulated the beam intensities of the oscilloscopes. The stimulating electrodes were connected to a stimulator of the hard valve type, which generated square wave pulses of any desired magnitude and duration either singly or repetitively. Provision was made to connect either these electrodes or the mass electrodes to a 45 volt DC source through a key. The bath was drained each time before readings were taken, except when the mass electrodes were employed.

[1] The authors wish to thank the National Health and Medical Research Council for a grant to carry out this work.

In check experiments (see later), the brain of a toad was destroyed, artificial respiration was administered, and the skin over a sartorius was removed. The pelvic end of the muscle was stimulated *in situ*, and recording electrodes were placed in light contact with the tibial end of the muscle. The drug was injected into the peritoneal cavity and E.C.G.'s were taken during the experiment. At the end of an experiment the aorta was cut to check that the blood was still circulating.

In confirmatory experiments (see later), a sartorius was mounted vertically in an organ bath, with the lower end fixed and the upper end attached to an isotonic lever writing on a smoked drum. Stimulation was effected while the muscle was bathed in fluid, via wires connected to the hooks attaching both ends of the muscle.

RESULTS.

Just maximal shocks, usually 3·5 volts at 1 millisecond duration were applied, and the tension and action potential were recorded. The bath was then filled with Ringer's solution to which yohimbine had been added to give a yohimbine concentration of 1 in 2,000. The bath was drained every ten minutes and the preparation was stimulated. After twenty to thirty minutes it was found that both tension and action potential had decreased. The stimulus was then increased to 10 volts at 1 millisecond duration, and the action potential and tension were restored to almost their original magnitude.

Readings at further ten minute intervals showed a progressive diminution in tension and action potential.

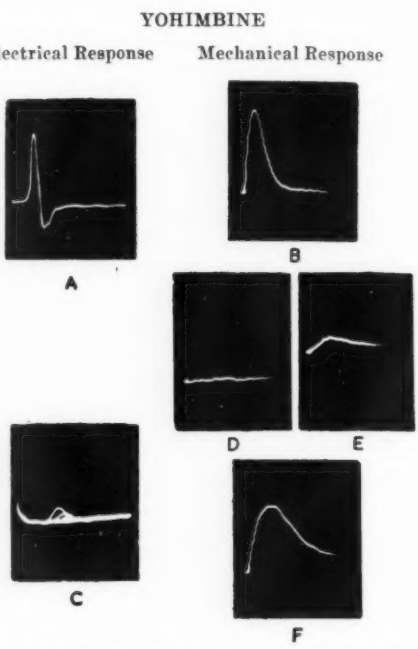

Fig. 1. Yohimbine. A. and B. control. 10 v. 1 msec. C. and D. 70 minutes later (repetitive stimulation 50 per second). E. 45 volts on end electrodes. F. 45 volts mass electrodes.

Repetitive stimulation at 10 volts at 1 millisecond and ½ second intervals showed a phenomenon in which action potentials of progressively smaller amplitude and slower velocities were initiated by each stimulus. A rest of ½ to 2 seconds would restore the potentials and velocities to their previous size. This phenomenon became more marked as the treatment with yohimbine progressed, until at 70 to 80 minutes repetitive stimuli produced one small spike only. Further treatment obliterated even this single spike, usually within 5 to 10 minutes.

The stimulating electrodes were then connected to the source of D.C. and the pick-up electrodes were removed. Brief stimulation (momentary tap on a key) then produced a slight increase in tension, or no change. Observation of the muscle showed a bunching of the fibres between the electrodes, extending down the muscle for a distance which varied with the potential of the stimulus, and a stretching of the remainder of the tissue. With 45 volts the spread was to about ¼ of the length. With this high potential, the tissue between the electrodes soon became inactive, but the bunching of muscle outside this area still was observable.

The direct current source was then transferred to the two longitudinal electrodes, and the bath was kept filled with yohimbine solution. When the key was closed for about 1/5th second at 45 volts, a contraction ensued, reaching between ½ and 2/3 of the tension developed in a normal muscle (Fig. 1).

The shape of the tension curve was different from that of a normal twitch. There was a slower development of tension, the maximum tension in a normal muscle being attained in about 2/3 of the time. The subsidence of tension was also slower, and merged into a "tail" which took up to 3 seconds before resting tension was restored. A maintained D.C. (10 seconds) stimulus gave a prolonged tension which subsided when the current was stopped. During the application of the current there was a gradual decrease in the tension, which corresponded with a decrease in the stimulating current, due to polarisation. Reversal of the current restored the tension to its original peak.

In the check expermients, using an animal in which the circulation and respiration were maintained, 10 mg. of yohimbine per 100 gm. of toad produced in about 40 minutes an effect on the conducted electrical wave in sartorius identical with that seen in the isolated muscle. Control experiments in both the isolated muscle, and the muscle *in situ*, showed no change in response to a single or repetitive stimuli over periods of three hours.

The experiment on isolated sartorius was repeated, using Clark's frog Ringer solution containing eleven times the usual potassium concentration. The action potential, velocity of conduction, and tension diminished solely with time of treatment. Repetitive stimulation did not produce the potential and velocity changes seen with yohimbine. The tension curve with mass electrodes did not exhibit the prolonged "tail" observed with yohimbine (Fig. 2).

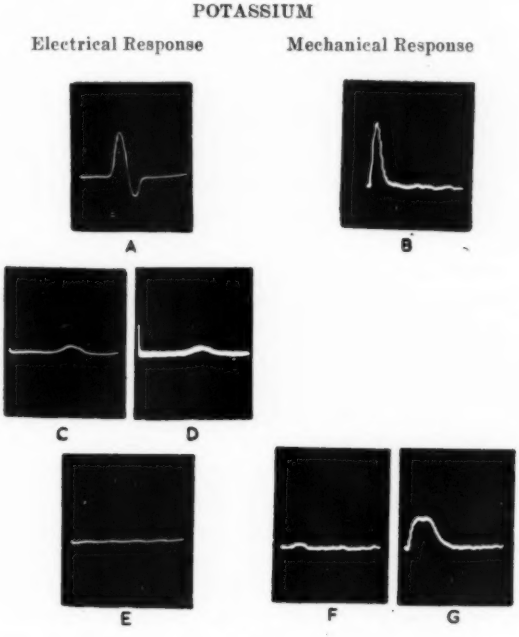

Fig. 2. Potassium. A. and B. Control. 3·5 v. 1 msec. C. 15 minutes later. D. 15 minutes later (repetitive 50 per second). E. 50 minutes later. F. 45 volts on end electrodes. G. 45 volts on mass electrodes.

In the confirmatory experiments, using the organ bath and isotonic recording, action potentials were not examined. A concentration of potassium eleven times normal produced a gradual reduction of tension until the muscle failed to respond to either single or tetanic stimuli. 22½ volts D.C. then gave a contraction which was larger than the original maximal twitch for a single stimulus. The contraction remained during the passage of the current, in fashion similar to a tetanic contraction, except that the tension developed was not so steady.

DISCUSSION.

In the muscle used, it might be argued that the action potential arises from stimulation of nerve and/or conduction along the nerve fibres, as well as from the muscle. In the species of toad used, nerve fibres may be traced to within a short distance of the pelvic end, as well as the tibial end of the muscle. However, the very slow rate of conduction of the muscle spike—three to four metres per second, indicates that the nerve fibres play little or no part in the action potential as their rate of conduction is greater. From the relative proportions of muscle and nerve fibres present in cross section, the nerve fibres would make little contribution to any action potential generated. Young (1941) has concluded that, in a whole, parallel-fibred muscle, the action potentials registered arise from the muscle, and not the nerve endings, and that the action potential of a single muscle fibre is qualitatively similar to that of whole muscle.

The effects of yohimbine on the muscle follow very closely the earlier results obtained for nerve (MacCallum and Shaw, 1949). There is a marked increase in the threshold, with a decrease in velocity, and amplitude of the action potential. With repetitive stimulation, the amplitude may fall to zero; however it recovers again in a few seconds. The exact cause of the delayed recovery is not known and the expression lengthened refractory period would be equally applicable. The action is much quicker on muscle, the same result often being achieved in one-third the time. Complete suppression of the action potential may be attained with prolonged bathing in yohimbine.

A criticism that could be levelled against the experiment is that the lowered supply of metabolites to the muscle contributed to the delayed recovery of the action potential to normal after repetitive stimulation. But an untreated muscle in the bath does not exhibit the phenomenon over a period of several hours. Also, recordings made while the sartorius remained *in situ*, and with the animal artificially respired, showed that yohimbine 10 mg. per 100 gm. body weight was effective in producing the same phenomenon of delayed recovery. At 2:1 or 3:1 heart block occurred after the yohimbine was injected, but the circulation was still maintained. Potassium in high concentration causes a gradual diminution in height of the conducted spike, with final obliteration, but no evidence of the delayed recovery seen with yohimbine has been observed. The same effect occurs when potassium in high concentration is used on nerve.

It thus seems likely that different parts of the mechanism of conduction are interrupted by each substance. The fact that the action of each on muscle and nerve conduction shows close parallels indicates that there are at least some elements in conduction common to both muscle and nerve.

Several authors have produced experimental evidence indicating that conduction and contraction in muscle can be separated.

Bishop and Kendall (1929) examined the action of formalin on a slow-acting muscle, the retractor penis of the turtle. They found the action potential separated into two elements, one of which they considered to be the excitation wave, and the other to be associated with the actual contractile processes in the muscle.

Gelfan (1930), using pore electrodes, observed local and graded mechanical responses in single muscle fibres in the absence of conduction.

Brown (1936) observed that the twitch produced by rapid decompression of a muscle was unaccompanied by any propagated action potential.

In the present experiments, the propagated excitation wave appears to have been abolished, while leaving the contractile processes of the muscle still active—as shown by the response to D.C. of high voltage.

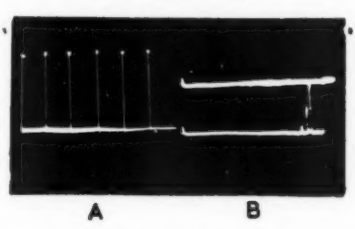

Fig. 3. Calibration. A. 10 milliseconds between vertical lines. B. 10 mvolts. between horizontal lines.

It was not found possible to look for action potentials when mass electrodes were used, as the muscle was immersed in solution, and the stimulating potentials would obliterate any action potential present. To eliminate the criticism that the threshold had been raised above the 10 volts at 1 millisecond used before transfer to battery, the D.C. stimulus was first applied to the stimulating electrodes at the pelvic end. A localized contraction resulted, spreading some distance from the electrodes. This spread was considered to be due to the spread of current passing between the electrodes. No evidence of any generalized contraction was obtained, indicating that the excitatory wave had been completely suppressed for all levels of stimulus. Stimulation with the mass electrodes then produced a generalized contraction of the muscle. The response could be repeated a number of times, indicating that it was not caused by a rigor, with death of the tissue.

The difference in the shape of the tension curve for yohimbine-treated, and potassium-treated muscles when stimulated, also indicates that the treatment has had some effect on the contractile elements of the muscle, even though the ability to contract remains. This is also supported by the fact that higher concentrations of yohimbine or treatment for much longer periods, destroys the ability of the muscle to contract, which indicates that yohimbine has also an effect on the contractile elements.

SUMMARY.

Yohimbine causes an increase in threshold and decrease in velocity and amplitude of the conducted electrical response in frog's sartorius muscle.

When the electrical response is no longer conducted the muscle is still able to contract when stimulated by mass electrodes.

Repetitive stimulation brings about an abolition of the electrical response, which however returns after about 2 seconds' rest. Similar effects were not obtained with increased potassium concentration.

REFERENCES.

Bishop, G. H. and Kendall, A. I. (1929): Amer. J. Physiol., 88, p. 77.
Brown, D. (1936): J. cell. and comp. Physiol., 8, pp. 141 and 315.
Gelfan, S. (1930): Amer. J. Physiol., 93, p. 1.
MacCallum, M., McCallum, I. A. N. and Shaw, F. H. (1949): Austral. J. exp. Biol., 27, p. 115.
Ramsey, R. W. and Street, S. F. (1941): Biol. Symp., Vol. III, p. 181.
Young, A. C. (1941): *Ibid.*, Vol. III, p. 153.